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Interactions between clonal asparagus plantlets and virulent and avirulent isolates of Fusarium.

Youn Su Lee
University of Massachusetts Amherst

INTERACTIONS BETWEEN CLONAL ASPARAGUS PLANTLETS AND VIRULENT AND AVIRULENT ISOLATES OF *FUSARIUM*

A Dissertation Presented

by

YOUN SU LEE

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Approved as to style and content by:

William J. Manning, Chair

Mark S. Mount, Member

Otto L. Stein, Member

Mark S. Mount, Department Head
Department of Plant Pathology

To

my parents, family members,

teachers, and friends.

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ABSTRACT

INTERACTIONS BETWEEN CLONAL ASPARAGUS PLANTLETS AND VIRULENT AND AVIRULENT ISOLATES OF *FUSARIUM*

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YOUN SU LEE, B.A., KANGWEON NATIONAL UNIVERSITY

M.S., LOUISIANA STATE UNIVERSITY

Ph.D., UNIVERSITY OF MASSACHUSETTS

Directed by: Professor William J. Manning

Fusarium oxysporum was isolated most frequently, followed by *F. moniliforme*, and *F. solani* from infected asparagus plants grown in the field.

In pathogenicity tests both with seedlings and plantlets, *F. moniliforme* showed slightly higher virulence than *Fusarium oxysporum* did in general. *Fusarium moniliforme* showed more consistent virulence on both seedlings and plantlets than *F. oxysporum* did. *Fusarium oxysporum* showed slightly higher virulence on plantlets than on seedlings. *Fusarium solani* showed very weak or no sign of virulence on seedlings and plantlets, respectively, in both tests.

In protection tests with plantlets, most protection of asparagus against virulent fusarial infections occurred when challenge isolates were inoculated five or seven days after inoculation of protective fusarial species. Avirulent *F. oxysporum* was a more effective protective agent against infection of *F. moniliforme* than it was against *F. oxysporum*.

Fusarium solani was more effective against infection of *F. oxysporum* than it was against *F. moniliforme*.

Virulent fusarial species showed significant increase in conidial populations on asparagus plantlet root and stem segments, and showed higher root and stem rot ratings than avirulent fusarial species did. Avirulent fusarial species showed minimal increase in conidial populations on both root and stem segments, and showed low to very low root and stem rot ratings.

All fusarial species infected asparagus plantlets through primary and lateral root tips, natural wounds, and between the walls of the epidermal cells directly. Some penetration was appressorium-like and direct. It was assumed that the meristematic region could act as a major infection site. Virulent fusarial species were growing faster and more abundantly inside and outside of the plantlet epidermal areas than were avirulent fusarial species. *Fusarium solani* was the slowest growing species. Within a short period, virulent fusarial species caused cortical rots. However, over extended periods, they invaded tracheary elements eventually, and caused extensive damages. Avirulent *F. oxysporum* accumulated heavily on and around the epidermal areas even if it invaded part of cortical cells inside the epidermal regions. *Fusarium solani* caused proliferation of lateral roots and increased the surface area of primary and secondary roots.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Asparagus (*Asparagus officinalis* L.) is a member of the family Liliaceae, and is a native species of the Mediterranean region, parts of Russia, and the British Isle (Ware and McCollum, 1975). People have been eating asparagus for more than 2,000 years (Halfacre and Barden, 1979) and it has been cultivated as a crop plant from at least the time of the Roman period. The spread of asparagus through Europe was partially due to Roman troops who established asparagus beds throughout the Roman empire. Asparagus was introduced to North America and other parts of the New World by early European settlers in the middle of nineteenth century (Sandsted *et al*., 1985). Asparagus is presently commercially cultivated throughout the world in countries such as Brazil, Taiwan, Canada, Mexico, the United States, Germany, and Great Britain; and is a highly valued vegetable crop by many people.

Asparagus plants are dioecious. The flowers are small, bell shaped, and whitish green. Male flowers are more conspicuous than female flowers. Following pollination of female flowers by bees and other insects, a berry, which has one to eight seeds and turns red at maturity, develops. The seeds, which are threshed from the berry when dry, are single, large, black, and generally round with one flattened side. Female plants are somewhat less productive and shorter lived

than male plants in general because of the energy allocated to seed production.

The underground portion of the plant consists of a network of rhizomes, fleshy storage roots, and fibrous roots. The fleshy roots as well as the spears are initiated from rhizomes. Together, the fleshy roots and rhizome make up the crown, which is the perennial portion of the asparagus plant. Fleshy roots serve not only as storage organs for the carbohydrates from the stem, but also as the site of fibrous root development. Fibrous roots, which live for one or two seasons, function in the absorption of water and nutrients from the soil. Asparagus spears are edible shoots that develop on rhizomes when the soil temperature is warm and the water supply is favourable. The spears, if not harvested, develop into stems 4-6 feet tall. Carbohydrates and other compounds necessary for plant growth and development are produced in the stems throughout the growing season. These substances are translocated to the fleshy roots, where they are stored and used to produce spears the following spring.

When the soil temperature is below approximately 50 F^{o} or the water supply is limited, crowns become dormant. In the Northeastern region of U.S.A., cool soil temperatures induce winter dormancy, whereas in tropical or subtropical growing areas, irrigation may be withheld to induce dormancy or special methods are employed to produce the crop without a dormant period. The dormant period preserves the

carbohydrates or energy, giving rise to spear production the following season.

Fusarium species are ubiquitous fungi found not only in temperate and tropical areas, but also in such diverse environments as the arctic and deserts. They are usually saprophytes but can also be pathogens that attack plants, animals and humans. Fusaria cause cortical rots, head blights, leaf spots, root rots, fruit rots, cankers, diebacks, and vascular wilt diseases of plants. These fungi attack a diverse group of plants including such crops as common flax (*Linum usitatissimum*), tomato (*Lycopersicon esculentum*), tobacco (*Nicotiana tabacum*), banana (*Musa* sp.), pea (*Pisum sativum*), sweet potato (*Ipomoea batatas*), lentil (*Lens culinaris*), cabbage (*Brassica oleracea*), cotton (*Gossypium* sp.,), muskmelon (*Cucumis melo* var. *reticulatus*), and watermelon (*Citrullus lanatus*). Crops such as carnation (*Dianthus caryophyllus*), chrysanthemum (*Chrysanthemum* sp.), daffodil (*Narcissus pseudonarcissus*), stock (*Matthiola incana*) and aster (*Aster* sp.), and tree such as date palm (*Phoenix dactylifera*) is also susceptible to attack by different *F. oxysporum* formae specialis. On asparagus plants, several *Fusarium* species such as *F. oxysporum*, *F. oxysporum* f. sp. *asparagi*, *F. moniliforme*, *F. roseum*, *F. solani*, and other *Fusarium* species cause disease symptoms on roots and crowns resulting in decline and death (Farr *et al.*, 1989).

Many species of *Fusarium* also attack the cortex, storage tissues or seeds (Inglis, 1980) of the host. The diseases

caused by these fungi, known by a variety of names including root rot, foot rot, stem rot, dry rot, scab, seedling blights, stand decline, and bulb rot, have, as their initial symptom, the development of small lesions on the affected plant part. Initial lesions may enlarge and cause girdling, which then results in the stunting, chlorosis, and collapse of the plant (Pennypacker, 1981). The cortical rot fusaria are soil-, air- or seed-borne and the mode of host penetration reflects this variation. The soilborne cortical-rotting fusaria generally infect roots or hypocotyls of the host by penetrating stomata, wounds, or directly through the root tip or meristematic region of the roots and through the hypocotyl (Bywarter, 1959; Chi *et al.*, 1964; Christou and Snyder, 1962; Hancock, 1968; Kamel *et al.*, 1973; Siddiqui and Halisky, 1968). Airborne conidia of *Fusarium* usually gain entry to their host through wounds and stomata (Dahl, 1934; Hadley, 1973; Phillips, 1962).

Damicone and Manning (1985) isolated *Fusarium* species from internal crown tissues of first-year asparagus grown from transplants. In their study, *F. moniliforme* and *F. oxysporum* were the main causal agents of the disease symptoms, with *F. moniliforme* showing higher virulence compared to that exhibited by *F. oxysporum*. *Fusarium solani* showed very low disease-causing capabilities. In a recent similar study with *Fusarium* species obtained from five-year-old field-grown asparagus, it was also found that *F. moniliforme* was more virulent than *F. oxysporum* on asparagus seedlings and *F. solani* was considered nonpathogenic to asparagus seedlings

(LaMonida and Elmer, 1988). It was also found that *Fusarium oxysporum* and *F. moniliforme*, isolated from diseased asparagus plants in the field, were the major causal agents of root and stem rot of asparagus plantlets (Lee and Manning, 1991a). In their study, *F. moniliforme* showed more virulence than *F. oxysporum* did, and *F. solani* was found to be non-pathogenic to asparagus plantlets *in vitro*.

Preinoculating with a *Cephalosporium* isolate, that was found to colonize the xylem vessels of tomato plants, delayed or prevented the development of symptoms of fusarial wilt in tomato seedlings (Chisler *et al.*, 1962). Other *Cephalosporium* spp. from soil showed the same inhibitory effect on wilt of cowpea (*Vigna unguiculata*) (Long, 1963). Tomato plants, 1-2 months old, grown in pots, were also partially protected against *F. oxysporum* f. sp. *lycopersici* by preinoculation with different isolates of *F. oxysporum* (Matta, 1966). Meyer and Maraite (1971) found that muskmelon plants inoculated with both virulent and less virulent strains showed delayed symptom appearance as compared to plants inoculated only with the more virulent strain. They explained the result in terms of competition which is probably most active at the penetration site.

Several experiments conducted in field plots showed that inoculation of gladiolus corms with certain isolates of *F. moniliforme 'Subglutinans'* Snyd. & Hans. and *F. solani* protected the corms from infection by the *Fusarium* yellows and corm rot pathogen, *F. oxysporum* f. sp. *gladiolus* (Massey)

Snyd. & Hans (Magie, 1980). In this study, protection with *F. moniliforme* isolate M-685 was equal to that obtained with benomyl (Benlate) corm dip treatment.

Cross-protection by the introduction of *F. oxysporum* f. sp. *dianthi* into host tissue of tomato, susceptible to *F. oxysporum* f. sp. *lycopersici*, was successful; however, the biocontrol agent was only effective when it was applied a few days before a challenge inoculations with the pathogen (Wymore and Baker, 1982). In their study, protection, manifested as a reduction in symptom expression, was apparent after 24 days when *F. oxysporum* f. sp. *dianthi* was used as the biocontrol agent but not 34 days after the challenge inoculation. Resistance to cucumber wilt, caused by *Fusarium oxysporum* f. sp. *cucumerinum*, was induced in cucumber plants growing on a mineral agar medium by inoculation of the medium with other *F. oxysporum* formae specialis not pathogenic to cucumber (Gessler and Kuc, 1982).

Ogawa and Komada (1986) obtained some isolates of *F. oxysporum* from healthy sweet potato tissues and showed cross-protection effects against the wilt disease when they were inoculated into sweet potato sprouts before being planted into infested soil. The isolates were nonpathogenic to sweet potato and several species of major vegetable crops such as cucumber, bottle gourd, melon, Japanese radish, cabbage and tomato.

Paulitz *et al.* (1987) isolated nonpathogenic *F. oxysporum* from surface-disinfested, symptomless cucumber roots grown in

nonautoclaved soils. Those nonpathogenic isolates were effective enough to reduce the infection rate against virulent *F. oxysporum* f. sp. *cucumerinum* at all inoculation densities.

Louter and Edgington (1990) reported cross-protection as a method of controlling *Fusarium* crown and root rot of tomato caused by *F. oxysporum* f. sp. *radicis-lycopersici*. In their study, four isolates of *F. oxysporum* and two isolates of *F. solani,* avirulent to tomato, significantly reduced root rot by as much as 87%.

Mandeel and Baker (1991) introduced two nonpathogenic strains of *F. oxysporum* into soil and obtained decreased slope values of inoculum density-disease incidence curves generated for *Fusarium* wilt of cucumber induced by *F. oxysporum* f. sp. *cucumerinum*. They explained the mechanisms involved in the control as competition in the rhizosphere and infection sites, as well as induction of enhanced resistance in the host. (Lee and Manning (1991b) achieved reduction of root and crown rot of tissue-cultured asparagus plantlets by prior inoculation with an avirulent isolate of *F. oxysporum in vitro*.

Cortical rot diseases are caused by several species of *Fusarium*. These fungi are almost entirely confined to infecting and destroying the cortical regions of their hosts. Only during the late stages of pathogenesis do some of these pathogens invade the vascular tissues. Once in the host, these fusaria are generally intercellular, at least initially (Christou and Snyder, 1962; Hancock, 1968; Sparnicht and Roncadori, 1972).

The majority of the cortical fusaria become intracellular in the cortical cells during the later stages of pathogenesis (Adams, 1921; Pugh *et al*., 1933; Hadley, 1973; Phillips, 1962). Some members of this category of fusaria only move intracellularly when the host cells are dead or dying, as is the case with *F. solani* f. sp. *phaseoli* in bean (Christou and Snyder, 1962). Once the host cortex is colonized, frequent sporulation occurs. In contrast to the vascular wilt fusaria, the cortical rot fusaria rarely sporulate in the plant (Christou and Snyder, 1962; Pugh *et al*., 1933).

The colonization of the vascular system of infected plants by fungal wilt pathogens represents a unique adaptation. Until the advanced stages of the disease syndrome, the pathogen is confined almost exclusively to the xylem vessels (Green, 1981). The wilt fungi grow slowly in the early stages of host colonization, primarily in the vessel lumen. The mycelium grows from one vessel to another, mainly through pits. Later the fungi may follow the middle lamella intercellularly and eventually penetrate the cell wall directly. However, host colonization occurs much more rapidly in the vascular system than can be accounted for by mycelial growth alone.

Translocation of spores in the vascular system varies with both the host and the pathogen. In banana (Beckman *et al*., 1961), microconidia of *F. oxysporum* f. sp. *cubense* are carried only short distances in the sap stream before they lodge on a perforation plate or other obstruction. The spore

then germinates; the germ tube penetrates the obstruction, and fresh spores are produced beyond the obstruction (Green, 1981).

The pathological anatomy of plants infected with formae specialis of *F. oxysporum* will vary with the specific host. These hosts include carnation (Pennypecker and Nelson, 1972), cabbage (Smith and Walker, 1930), radish (Peterson and Pound, 1960), tomato (Chambers and Corden, 1963), banana (Beckman *et al.*, 1961; Trujillo, 1963), sweet potato (McClure, 1950), and mimosa tree (Phipps and Stipes, 1976).

Beckman *et al.*, (1961) found that the extent of vascular invasion following wound inoculations of Gros Michel banana roots with spore-suspensions of *Fusarium oxysporum* f. sp. *cubense* was limited in upper (young) and extensive in lower (older) roots, and they concluded that physical traps or barriers could retard the advance of the pathogen until host-response resistance mechanisms become operative.

Graham (1955) found that *Fusarium* species which cause infection on asparagus penetrate directly into the embryonic region of the root tip, or through stomata on the hypocotyl, and colonizes the host both intercellularly and intracellularly. Its limitation to the cortex is regarded as unusual among parasitic fusaria, which are mainly vascular.

The mode of penetration and the host-parasite relationships of *F. solani* f. *phaseoli* were studied (Christou and Snyder, 1962). They found that the fungus penetrates both roots and hypocotyls, not forming appressoria but producing a

small thallus. It enters the plant directly or through mechanical or natural wounds, but most commonly through stomata of the hypocotyl.

Histological studies showed that penetration and subsequent development of *F. avenaceum*, *F. oxysporum*, and *F. solani* were similar in alfalfa and red clover (Chi *et al.*, 1964). They found all three species entered uninjured epidermal cells of roots, seed coats, and cotyledons by direct penetration without the formation of appressoria. Penetration was both intercellular and intracellular. Most abundant penetration occurred in the meristmatic tissues, but the regions of cell enlargement and differentiation also were invaded. The cortex was completely colonized. All 3 species entered the xylem, but *F. solani* was limited mostly to the epidermal and cortical areas. Root tips were penetrated by the time seedlings were 2 days old. Seed coats of germinating seeds were quickly penetrated and colonized by all three fungi. Abundant hyphae were found in the cotyledons, leaf primordia, and young shoots. They also found similar fungal development on naturally infected and artificially inoculated plants.

Studies concerning asparagus such as those of Robins (1924), and Jones and Rosa (1928) have dealt chiefly with the cultural and commercial aspects rather than morphological or morphological-histopathological ones. Borthwick (1925), Robbins and Borthwick (1925), Cooley (1895), and Blasberg (1932) have used morphology largely as a basis for

physiological study. Some of these have dealt with limited phases, Borthwick (1925), and Robbins and Borthwick (1925) with germination; Cooley (1895) with reserve cellulose of the seed. Blasberg (1932) has given some attention to anatomy. His work included growth habit, seedling development, root structure, and stem structure. Blasberg (1932) worked on phases of the anatomy and explained anatomical aspects of many different species of asparagus, Mullendore (1935) worked on the anatomy of the seedling of asparagus extensively and elaborated each stages of development. Cheadle and Kosakai (1971) worked on vessels in Liliaceae and mentioned briefly the vessel elements of asparagus. Tomlinson (1965) also worked on the anatomy of Liliaceae and drew comparisons with that of Eriocaluaceae. Ghouse and Yunus (1973) found ectodesmata formation in the epidermal cell walls of asparagus in their anatomical work.

In recent years, there have been no further studies on pure asparagus anatomy or anatomy in relation to morphological changes. Instead, anatomical work on asparagus was combined with other research interest areas, especially with plant pathology. Some of the work on the anatomy of asparagus in relation to plant pathogenic fungi were published (Grove, 1976b; Sutherland *et al.*, 1989).

There are no effective strategies available against *Fusarium* disease on asparagus. There are no effective resistant varieties available, and chemical control has met with limited success (Lacy, 1977; Wiebe, 1967). Also, there

were few reports of successful biological management of *Fusarium* disease on asparagus. Therefore, based on previous reports on stem, crown and root rot of asparagus in Western Massachusetts (Gilbertson, 1981), and on biological management of *Fusarium* disease on asparagus with antagonistic soil microorganisms (Damicone, 1980) or avirulent *Fusarium oxysporum* (Manning, 1983), a study was undertaken with objectives as described in the following.

1.1 Objectives of the Study

1. Isolate *Fusarium* species from field grown asparagus plants.
2. Perform pathogenicity tests and to compare susceptibility of seedlings and plantlets to different *Fusarium* species such as avirulent *F. oxysporum* previously obtained from bean hypocotyl (Manning, 1983) and other *Fusarium* species isolated from field grown asparagus plants.
3. Determine the possible use of avirulent *Fusarium* species to protect against infection on asparagus plantlets by virulent fusaria and to determine the time between induction and challenge inoculation that gives the highest levels of protection through *in vitro* tests with plantlets.
4. Determine the different infectivity of virulent and avirulent *Fusarium* species on asparagus plantlets.

5. To study the histopathology of the infection process of virulent and avirulent *Fusarium* species within a short period of time and over extended periods.

CHAPTER 2

ISOLATION OF FUSARIAL SPECIES AND PATHOGENICITY TESTS WITH ASPARAGUS SEEDLINGS AND PLANTLETS

2.1 Introduction

Fusarium species associated with diseased asparagus were first reported in Massachusetts by Stone and Chapman (1908), who isolated a *Fusarium* species from young, wilted asparagus shoots. Cook (1923) reported a similar disease of asparagus caused by unidentified *Fusarium* sp. from New Jersey, and he called the disease "dwarf asparagus." Boyd (1930 and 1942) reported fusaria associated with diseased asparagus in Massachusetts, while Armstrong (1930) described a very similar fusarial disease in other parts of U.S.A.

Cohen and Heald (1941) first described the causal agent of the wilt and root rot disease of asparagus to be *Fusarium oxysporum* (Schlect) var. *asparagi*. The pathogen caused root rot and wilt symptoms on host plants in Washington. Reports on asparagus diseases found in the United States in 1921, 1924, 1935, 1936, and 1937 mention a stem rot caused by *Fusarium* to occur in Massachusetts, New York, New Jersey, and California (Cohen, 1946). Cohen and Heald (1941) reported that the fungus was able to initiate a symptom complexes in the host, and colonize roots, crowns, and stems. They

hypothesized that the pathogen survived as a saprophyte on senescing asparagus tissue during the winter and spring, and attacked living tissue in the summer.

Graham (1955) reported a *Fusarium* seedling blight of asparagus in Ontario. Affected seedlings appeared stunted, yellowed, and wilted; and some died. Graham identified the causal agent of the blight to be *F. oxysporum* Schlect. emend. Snyder and Hansen var. *redolens* (Wr.) Gordon, and to a lesser extent *F. moniliforme*. Both pathogens were restricted to cortical tissues of asparagus seedlings. *Fusarium oxysporum* var. *redolens* effectively colonized crown, root, and shoot cortical tissues, while *F. moniliforme* caused root tip necrosis. *Fusarium moniliforme* was isolated from seeds and stalks of asparagus plants in the field, but was rarely isolated from diseased seedlings in the field.

Grogan and Kimble (1954 and 1959) showed *F. oxysporum* f. sp. *asparagi* to be primarily responsible for the asparagus decline and replant problem in California. Lewis and Shoemaker (1964) tested asparagus seed lots from New Jersey, Massachusetts, Michigan, and Ontario for the presence of *F. oxysporum*. Plants from all seed lots showed a 20-100% infection rate with *F. oxysporum* f. sp. *asparagi*, and the pathogen was isolated from root lesions. Van Bakel and Kerstens (1970) described an asparagus disease caused by *F. oxysporum* f. sp. *asparagi*. Symptoms of the disease caused by the pathogen included small, brown, oval shaped lesions on

storage roots and lower stems. Plants declined due to a foot rot of stems, roots, and crowns.

Fusarium moniliforme was found to be associated with crown rot of asparagus in California (Endo and Burkholder, 1971). Almost all infected plants (93%) yielded *F. moniliforme* while 22% yielded *F. oxysporum*. Both pathogens were isolated from red-brown lesions on crowns, stems, and storage roots. *Fusarium moniliforme* was additionally isolated from commercial asparagus seed, volunteer seedlings, and plant debris. They considered *F. moniliforme* as a major factor in asparagus crown rot in California.

Both *F. oxysporum* and *F. moniliforme* were found to be associated with declining asparagus plants in Washington by Grove (1976a and 1976b). *Fusarium oxysporum* was the prevalent species isolated. It was found that *F. moniliforme* and *F. oxysporum* to be responsible for asparagus decline in Western Massachusetts (Blacklow and Manning, 1976). *Fusarium oxysporum* was the major causal agent in their study. Both pathogens caused asparagus seedling death, and root and stem lesions on mature plants. Root and crown rot caused by *F. oxysporum* and *F. moniliforme* is primarily responsible for asparagus decline in Michigan (Lacy, 1977; Hartung *et al.*, 1990), and New Jersey (Johnston *et al.*, 1979). *Fusarium oxysporum* f. sp. *asparagi* was isolated from discolored vascular root tissue and cortical root lesions, while *F. moniliforme* was primarily isolated from cortical stem and crown lesions. *Fusarium moniliforme* was found to be the main

casual agent of stem and crown rot disease while *F. oxysporum* was found to be the casual agent of root rot and wilt symptoms. *Fusarium moniliforme* and *F. oxysporum* f. sp. *asparagi* was also found to be associated with asparagus seed in Washington (Inglis, 1978; Inglis, 1980).

In Western Massachusetts, *F. oxysporum* and *F. moniliforme* was found to be causal agent of asparagus root, crown and stem rot in commercial asparagus beds. *Fusarium oxysporum* parasitize storage and feeder roots, cortical tissue of stem bases, and vascular crown tissues; and exist in soil as saprophyte, as chlamydospores or in association with volunteer plants or weed hosts, while *F. moniliforme* attacks aboveground plant parts and survives on seeds, volunteer plants, and in association with asparagus miner flies (Damicone, 1980; Damicone and Manning, 1985; Gilbertson, 1981). *Fusarium oxysporum* and *F. moniliforme* were also found to cause wilt and root rot of commercial asparagus in Taiwan (Tu, 1979).

Nigh (1985) established that pathogenic variability on asparagus plant exists between the fusarial isolates from different geographic areas which are highly diverse in cultural practices, soil type, climatology and periods of harvest.

LaMondia and Elmer (1988) isolated *Fusarium moniliforme*, *F. oxysporum* and *F. solani* from symptomatic and asymptomatic feeder roots, storage roots, crown and basal stem segments. In their study, they found that *F. moniliforme* was more virulent than *F. oxysporum* on asparagus seedlings and *F.*

solani was considered nonpathogenic, and they concluded that virulence on asparagus was a common trait with few exceptions among genetically distinct populations of *F. moniliforme* and *F. oxysporum* colonizing asparagus.

Hartung *et al.* (1990) found that 38% isolates of *Fusarium oxysporum* that were recovered from fields of all ages incited lesions on asparagus seedlings, *F. moniliforme* was recovered more frequently from plant debris than sieved soil, and all isolates of *F. moniliforme* were virulent. In Western Massachusetts, Lee and Manning (1991a) found that *F. oxysporum* was isolated more frequently than *F. moniliforme* from diseased plants grown in the field, and *Fusarium moniliforme* and *F. oxysporum*, isolated from diseased asparagus plants grown in the field, were the major causal agents of root and stem rot of asparagus seedlings and plantlets *in vitro*.

In this study, therefore, attempts were made to isolate *Fusarium* species, pathogenic and non-pathogenic (and/or possibly protective) to asparagus seedlings and plantlets, from asparagus plants grown in the field, to perform pathogenicity tests with seedlings and plantlets, and to compare susceptibility of seedlings and plantlets from infections of different fusarial species.

2.2 Isolation of Fusarial Species from Infected Asparagus Plants Grown in the Field

2.2.1 Materials and Methods

Asparagus plants showing typical crown and root rot symptoms were obtained from several locations in Amherst, MA and Sunderland, MA. For chlamydospore formation, edges of crown and root rot lesions were cut out and surface sterilized with 10% Clorox bleach (v/v) for 3 minutes and rinsed three times with sterile distilled water before plating on 2% water agar medium. Komada's medium (Komada, 1975) was chosen for selective isolation of *Fusarium oxysporum*, and KCl medium (Fisher *et al.*, 1983) was used for conidial chain formation by *Fusarium moniliforme*. Carnation leaf agar (carnation leaves on 2% water agar) (Burgess *et al.*, 1988; Fisher *et al.*, 1982), for conidia and conidiophore formations, was used for accurate identification. Also, different colors of fungal growth on PDA medium (Nelson *et al.*, 1983) among different species of *Fusarium* were used as criteria for identification. Isolation of *Fusarium* species from original plates, and subsequent transfers were made by using single spore isolation technique (Nelson *et al.*, 1983) to eliminate most of the problems associated with variability and difficulty of identification. Final identification of each species was based on the taxonomic key by Nelson *et al.* (1983). Selected isolates were preserved in silica (Windells *et al.*, 1988) for further experiments.

2.2.2 Results and Discussion

Seventeen isolates of *F. oxysporum*, three isolates of *F. moniliforme*, one isolate of *F. solani*, and thirteen isolates of unknowns were obtained from a total of thirty-four selected subculture plates (Table 2.1). An avirulent *F. oxysporum* (AVFO) isolate was previously obtained from bean hypocotyl surfaces (Manning, 1983).

Fusarium oxysporum was isolated most frequently (50%), followed by *F. moniliforme* (8.8%), and *F. solani (2.9%)*. These results indicate the difference of saprobic capabilities among three different *Fusarium* species affecting asparagus in the field. They support previously reported results by Gilbertson (1981), Manning (1983), and Damicone and Manning (1985) in Western Massachusetts, and LaMondida and Elmer (1988) in Connecticut area.

2.3 Pathogenicity Tests with Seedlings and Plantlets *In Vitro*

2.3.1 Materials and Methods

2.3.1.1 Pathogenicity Tests with Seedlings

Seeds of asparagus clone Mary Washington were surface-sterilized with 25% benomyl in acetone (100%) for overnight followed by washing with sterile distilled water three times to remove benomyl residues (Damicone *et al.*, 1981). Washed seeds were treated with 10% Clorox bleach (v/v) for a few

Table 2.1 *Fusarium* species isolated from root and stem pieces of asparagus plants grown in the field.

ID. #	Fungal species	ID. #	Fungal species
AS17	*F. oxysporum* 17	AS34	Unknown
AS18	*F. solani*	AS35	*F. oxysporum* 35
AS19	*F. oxysporum* 19	AS36	*F. oxysporum* 36
AS20	Unknown	AS37	*F. moniliforme* 37
AS21	*F. oxysporum* 21	AS38	*F. oxysporum* 38
AS22	*F. oxysporum* 22	AS39	Unknown
AS23	*F. oxysporum* 23	AS40	Unknown
AS24	*F. moniliforme* 24	AS41	Unknown
AS25	*F. oxysporum* 25	AS42	Unknown
AS26	*F. oxysporum* 26	AS43	Unknown
AS27	*F. oxysporum* 27	AS44	Unknown
AS28	*F. moniliforme* 28	AS45	*F. oxysporum* 45
AS29	Unknown	AS46	Unknown
AS30	Unknown	AS47	*F. oxysporum* 47
AS31	*F. oxysporum* 31	AS48	*F. oxysporum* 48
AS32	Unknown	AS49	*F. oxysporum* 49
AS33	Unknown	AS50	*F. oxysporum* 50

minutes and washed with sterile distilled water three times. Cleaned seeds were soaked for 24 hours in sterile distilled water to facilitate seed germination. Soaked seeds were then blotted on sterile filter paper before plating on 0.6% water agar for 2 weeks for germination and initial growth. Germinated seedlings were aseptically transplanted on Hoagland solution (Hoagland and Arnon, 1950) slants (25 mm test tubes) and were established on the growth bench for 3 days at room temperatures. Two agar discs (control) or two agar discs bearing a *Fusarium* isolate, were placed in contact with roots, just below the crown. Four isolates of *Fusarium oxysporum* (Isolate 19, 45, 48, and 49), three isolates of *F. moniliforme* (Isolate 24, 28, and 37), two avirulent *F. oxysporum* (AVFO) isolates (Isolate AVFO-1 and AVFO-2) obtained previously from bean hypocotyl surfaces (Manning, 1983), and one isolate of *F. solani* were used as inocula. Seedlings were evaluated for disease incidence four weeks after the inoculation. The experiment was repeated twice.

2.3.1.2 Pathogenicity Tests with Plantlets

Asparagus plantlets (Female clone, NJ362M) were obtained through meristem tip culture (Kahn 1976; Murashige *et al.*, 1972; Reuther, 1984), rooted on rooting medium, increased on multiplication medium (Desjardins *et al.*, 1987; Yang, 1977; Yang and Clore, 1973; Yang and Clore, 1974a; Yang and Clore, 1974b; Yang and Clore, 1975), and placed on filter paper

slants in test tubes (25 mm) containing Hoagland solution. Two agar discs without inoculum (control) or two agar discs containing a *Fusarium* isolate, were placed in contact with roots, just below the crown. Four isolates of virulent *F. oxysporum* (Isolate 19, 45, 48, and 49), three isolates of *F. moniliforme* (Isolate 24, 28, and 37), two avirulent *F. oxysporum* (AVFO) isolates (Isolate AVFO-1 and AVFO-2) previously obtained from bean hypocotyl surfaces (Manning, 1983), and one isolate of *F. solani* were used as inocula. Plantlets were evaluated for disease incidence 4 weeks after inoculation. The experiment was repeated twice.

2.3.2 Results and Discussion

2.3.2.1 Pathogenicity Tests with Seedlings

In the first test (Table 2.2), seedlings inoculated with all isolates of *F. moniliforme* and *F. oxysporum* showed severe stunting of growth, and showed significant differences from seedlings treated with agar plugs without fusarial species (control), and from seedlings inoculated with avirulent *F. oxysporum* (AVFO) or *F. solani*. *Fusarium moniliforme* and *F. oxysporum* did not show significant differences in disease incidence between the two. However, *F. moniliforme* caused slightly more severe stunting of seedlings than *Fusarium oxysporum* did. Even though there were statistically significant differences from controls, avirulent *F. oxysporum* (AVFO) isolates and *F. solani* isolates caused mild disease

Table 2.2 Results of first *in vitro* pathogenicity test with asparagus seedlings[a]

	Replications[b]				
Iso[c] \	1	2	3	4	Ave[d]
AVFO-1	1	1	1	1	1.0b
AVFO-2	1	1	1	1	1.0b
FM 24	3	3	3	4	3.3a
FM 28	3	4	4	5	4.0a
FM 37	4	4	4	4	4.0a
FO 19	4	3	3	4	3.5a
FO 45	4	3	4	3	3.5a
FO 48	4	3	3	3	3.3a
FO 49	3	4	3	4	3.5a
FS	1	2	1	1	1.3b
CTR[e]	0	0	0	0	0c
LSD (P=0.05)					0.67

[a]Results of each replication were the mean of four observations. [b]Disease ratings were based on 0-5 scale where 0=no disease, and 5=death of seedlings. [c]Iso=isolates as follows; AVFO=avirulent *F. oxysporum*, FM=*F. moniliforme*, FO=virulent *F. oxysporum*, and FS=*F. solani*. [d]Ave=average. Means with the same letter are not significantly different at P=0.05. [e]CTR=control.

symptoms occasionally with increased root discoloration. *Fusarium solani* isolates caused severe root discoloration, no severe disease symptoms, and appeared to mildly stimulate seedling growth by showing increased seedling vigor.

In the second test (Table 2.3), the results were similar as those observed in the first test. However, isolates of *F. oxysporum* and *F. moniliforme* caused slightly more severe symptoms in appearance than those observed in the first test, and isolates of *F. moniliforme* killed all tested seedlings in the second test.

2.3.2.2 Pathogenicity Tests with Plantlets

All plantlets inoculated with *F. moniliforme* isolates were killed in both tests (Tables 2.4 and 2.5). Isolates of virulent *F. oxysporum* killed some plantlets, but caused only moderate disease in others for both tests with plantlets.

In the first test (Table 2.4), there were significant differences between plantlets inoculated with all isolates of *F. moniliforme* or *F. oxysporum* isolates 48 and 49, and control plantlets, and there were no significant differences between plantlets inoculated with avirulent *F. oxysporum* (AVFO) isolate and control plantlets. Also, there were no significant differences between plantlets inoculated with *F. solani* and control plantlets. Isolates of *F. solani*, *F. moniliforme*, and *F. oxysporum* caused moderate to very severe root discolorations. Avirulent *F. oxysporum* isolates (AVFO)

Table 2.3 Results of second *in vitro* pathogenicity test with asparagus seedlings[a]

	Replications[b]				
Iso[c] \	1	2	3	4	Ave[d]
AVFO-1	1	2	1	1	1.3b
AVFO-2	1	1	2	1	1.3b
FM 24	5	5	5	5	5.0a
FM 28	5	5	5	5	5.0a
FM 37	5	5	5	5	5.0a
FO 19	4	5	5	4	4.5a
FO 45	5	5	4	3	4.3a
FO 48	5	5	4	3	4.3a
FO 49	5	5	5	5	5.0a
FS	1	2	1	1	1.3b
CTR[e]	0	0	0	0	0c
LSD (P=0.05)					0.74

[a]Results of each replication were the mean of four observations. [b]Disease ratings were based on 0-5 scale where 0=no disease, and 5=death of seedlings. [c]Iso=isolates as follows; AVFO=avirulent *F. oxysporum*, FM=*F. moniliforme*, FO=virulent *F. oxysporum*, and FS=*F. solani*. [d]Ave=average. Means with the same letter are not significantly different at P=0.05. [e]CTR=control.

Table 2.4 Results of first *in vitro* pathogenicity test with asparagus plantlets[a]

\Rep[b]	Root Rot Rating Means[c]					Root Discoloration Rating Means[d]				
Iso[e]\	1	2	3	4	Ave[f]	1	2	3	4	Ave[f]
AVFO-1	1	0	1	1	0.8c	1	1	3	2	1.8d
AVFO-2	1	0	0	1	0.5c	1	2	1	2	1.3d
FM24	5	5	5	5	5.0a	5	5	5	5	5.0a
FM28	5	5	5	5	5.0a	5	5	5	5	5.0a
FM37	5	5	5	5	5.0a	5	5	5	5	5.0a
FO19	2	2	3	3	2.5b	4	4	3	4	3.8c
FO45	1	5	5	5	4.0a	5	4	5	4	4.5ab
FO48	5	5	5	5	5.0a	5	4	5	4	4.5ab
FO49	5	5	5	5	5.0a	5	5	5	5	5.0a
FS	0	0	0	0	0c	4	5	4	4	4.3bc
CTR[g]	0	0	0	0	0c	0	0	0	0	0e
LSD (P=0.05)			1.01					0.66		

[a]Results of each replication were the mean of four observations. [b]Rep=replications. [c]Root rot ratings were based on 0-5 scale where 0=no disease, and 5=death of plantlets. [d]Root discoloration ratings were based on 0-5 scale where 0= no color change, and 5=severe discoloration. [e]Iso=isolates as follows; AVFO=avirulent *F. oxysporum*, FM=*F. moniliforme*, FO=virulent *F. oxysporum*, and FS=*F. solani*. [f]Ave=average. Means with the same letter are not significantly different at P=0.05. [g]CTR=control.

Table 2.5 Results of second *in vitro* pathogenicity test with asparagus plantlets[a]

\Rep[b]	Root Rot Rating Means[c]					Root Discoloration Rating Means[d]				
Iso[e]\	1	2	3	4	Ave[f]	1	2	3	4	Ave[f]
AVFO-1	0	1	1	1	0.8c	2	3	3	2	2.5bcd
AVFO-2	0	1	1	0	0.5c	2	2	2	3	2.3cd
FM24	5	5	5	5	5.0a	5	5	5	5	5.0a
FM28	5	5	5	5	5.0a	5	5	5	5	5.0a
FM37	5	5	5	5	5.0a	5	5	5	5	5.0a
FO19	1	5	2	5	3.3b	1	3	2	3	2.3cd
FO45	1	5	5	5	4.0ab	1	2	2	3	2.0d
FO48	5	5	5	5	5.0a	2	3	3	4	3.0bc
FO49	5	5	5	5	5.0a	2	3	3	4	3.0bc
FS	0	0	0	0	0c	3	3	4	3	3.3b
CTR[g]	0	0	0	0	0c	0	0	0	0	0e
LSD (P=0.05)			1.35					0.80		

[a]Results of each replication were the mean of four observations. [b]Rep=replications. [c]Root rot ratings were based on 0-5 scale where 0=no disease, and 5=death of plantlets. [d]Root discoloration ratings were based on 0-5 scale where 0=no color change and 5=severe discoloration. [e]Iso=isolates as follows; AVFO=avirulent *F. oxysporum*, FM=*F. moniliforme*, FO=virulent *F. oxysporum*, and FS=*F. solani*. [f]Ave=average. Means with the same letter are not significantly different at P=0.05. [g]CTR=control.

caused slight root discoloration. All isolates of *Fusarium* species caused significant differences in root discoloration compared to those of control plantlets. However, there were no visible root discoloration differences between plantlets inoculated with avirulent *F. oxysporum* (AVFO) and those inoculated with pure agar plugs (control).

In the second test (Table 2.5), similar results were obtained as those observed in the first test. However, *F. oxysporum* isolate 19 showed slightly higher severity rating than that in the first test. In this second test, avirulent *F. oxysporum* (AVFO) isolates showed increased root discoloration than that observed in the first test. However, *F. solani* isolate and virulent *F. oxysporum* isolates showed decreased root discoloration ratings in this second test.

2.3.2.3 Comparison of Pathogenicity of *Fusarium* Species on Seedlings and Plantlets

Avirulent *F. oxysporum* (AVFO) isolates showed negligible virulence on seedlings even if there was a statistical difference when compared to control treatment (Tables 2.2 and 2.3). However, in pathogenicity test on plantlets (Tables 2.4 and 2.5), avirulent *F. oxysporum* (AVFO) did not show any difference from plantlets with control treatments. *Fusarium moniliforme* isolates caused severe to very severe root rot symptoms on both tests with seedlings and plantlets. *Fusarium moniliforme* isolates caused severe rot symptoms (Table 2.2) or

death of seedlings (Table 2.3), and all *F. moniliforme* isolates caused death of plantlets (Tables 2.4 and 2.5). Overall, *F. moniliforme* isolates showed slightly higher virulence on plantlets than on seedlings. Isolates of *F. oxysporum* showed slightly different pathogenicity on seedlings or plantlets with different treatments (Tables 2.2, 2.3, 2.4, and 2.5). Some *F. oxysporum* isolates caused severe rot symptoms on both seedlings and plantlets, and some caused death of seedlings and plantlets. However, *F. oxysporum* isolates showed higher virulence on plantlets than on seedlings. *Fusarium solani* isolates showed weak virulence on seedlings (Tables 2.2 and 2.3), and did not show any sign of virulence on plantlets in both tests (Tables 2.4 and 2.5).

Nigh (1985) showed that pathogenic variability on asparagus plant exists between the different fusarial isolates from diverse geographic areas. Also, different clones of asparagus used for seedling (Mary Washington) and plantlet (New Jersey) pathogenicity tests might caused minor differences in susceptibility of seedlings and plantlets to infections of different fusarial species.

In conclusion, however, it was found that isolates of *F. moniliforme* and *F. oxysporum* caused severe to highly severe disease symptoms on both seedlings and plantlets, whereas those of avirulent *F. oxysporum* (AVFO) and *F. solani* did not cause much damage or no damage at all on both seedlings and plantlets. Overall, there were no differences between seedlings and plantlets in susceptibility by virulent fusarial

infections. It was also found that there was no difference in infectivity of avirulent *F. oxysporum* (AVFO) and *F. solani* on both seedlings and plantlets.

CHAPTER 3

DETERMINATION OF TIME BETWEEN INDUCTION AND CHALLENGE INOCULATION THAT GIVES THE HIGHEST LEVELS OF PROTECTION THROUGH *IN VITRO* TESTS WITH PLANTLETS

3.1 Introduction

There are several described basic mechanisms of microbial protection of plant roots. These are competition for space and/or nutrients, production of antibiotics or plant growth regulators (Baker, 1968; Brown, 1972). One or two, or more of these mechanisms may be in operation at any one time.

Malta (1971) described the use of saprophytic or mildly pathogenic species, form species, or races of the pathogen for biological management of root rot diseases to protect the host against virulent forms. This so called "immunization" (Malta 1971), "induced resistance" (Malta, 1971), or "cross protection" (Graham, 1955; Schnathorst and Mathre, 1966) has been the focus of many studies involving *Fusarium* wilt diseases, but other diseases such as black shank of tobacco (McIntyre and Miller, 1978), *Verticillium* wilt of cotton (Schnathorst and Mathre, 1966), and virus diseases (Malta, 1971) have been managed by using this approach. This method uses a pre-inoculation with the mild saprophytic parasite, certain periods of incubation, and challenge inoculations.

The mild saprophyte colonizes the cortex and/or vascular system without causing severe symptoms and is able to ward off colonization by the pathogens.

There were many attempts at cross protection and success has been reported in the field and greenhouse. Bega (1954) and McClure (1951) protected sweet potato from wilt caused by *Fusarium oxysporum* with pre-inoculations with *F. solani*. When *F. solani* was used as a pre-inoculation, wilt could be reduced by 90-100 percent. Graham (1955) used mixed inoculations of asparagus with both *F. oxysporum* and *F. moniliforme* which resulted in reduced seedling blight than when either isolate was used alone. Pea wilt disease caused by *F. oxysporum* was reduced by prior or mild inoculation with *F. solani* in the greenhouse and field by Buxton and Perry (1959). They concluded that *F. solani* was a more aggressive colonizer of the root cortex which resulted in a hypersensitive reaction in the host tissue that prevented entrance by the wilt fungus.

Davis (1967) achieved protection against *Fusarium* wilts in axenic culture with pre-inoculation of the hosts such as tomato, carnation, flax, and cabbage with non-pathogenic formae specialis of *Fusarium oxysporum*. Davis (1968) also achieved partial control in the greenhouse with preinoculations of four different non-pathogenic isolates of the wilt fungus. Langton (1969) reported protection of tomato cuttings from *F. oxysporum* f. sp. *lycopersici* with mixed or pre-inoculations of a pea wilt isolate of the same species. He postulated that the successful control was due to the

colonization of the roots by the non-pathogen which resulted in mechanical exclusion or localized resistance. Meyer and Maraite (1971) achieved the same results with experiments on *Fusarium* wilt of muskmelon. These studies indicate possible cross-protection in *Fusarium* diseases.

Magie (1980) conducted several experiments in field plots that showed inoculation of gladiolus corms with certain isolates of *Fusarium moniliforme 'Subglutinans'* Snyd. & Hans. and *F. solani* protected the corms from infection by the *Fusarium* yellows and corm rot pathogen, *F. oxysporum* f. sp. *gladioli* (Massey) Snyd. & Hans.

Damicone and Manning (1982), and Manning (1983) developed methods to obtain avirulent isolates of *F. oxysporum* (Isolate 1B) from bean hypocotyl surfaces and to determine their efficacy in protecting asparagus seedlings in gnotobiotic culture and in naturally-infested soil in the greenhouse. In their study, isolate of 1B of *F. oxysporum* (nonpathogenic) was selected for trial in two commercial fields. Roots of 10-week-old seedlings were incubated in a spore suspension of 1B for 24 hours prior to planting. This resulted in significantly larger plants with reduced disease symptoms compared with the control. Treatment with 1B conferred protection comparable to that achieved with a 0.1% benomyl fungicide treatment.

Wymore and Baker (1982) reported cross-protection by the introduction of *F. oxysporum* f. sp. *dianthi* into the host tissue of tomatoes susceptible to *F. oxysporum* f. sp.

lycopersici. However, the biocontrol agent was only effective when it was applied a few days before a challenge inoculation with the pathogen. Nonpathogenic isolates of *Fusarium oxysporum* from cucumber (Paulitz *et al*., 1987), sweet potato (Ogawa, 1989; Ogawa and Komada, 1986), and tomato (Louter and Edington, 1990), were used as protection agents for the control of virulent fusarial infection on each different host.

Tu *et al*. (1990) used a saprophytic isolate of *Fusarium oxysporum* nonpathogenic to asparagus to reduce the severity of the disease in pot tests. Mandeel and Baker (1991) introduced two nonpathogenic *Fusarium oxysporum* into soil, and obtained reduced disease ratings on *Fusarium* wilt of cucumber induced by *F. oxysporum* f. sp. *cucumerinum*. Lee and Manning (1991b) achieved reduction of root and crown rot of tissue-cultured asparagus plantlets *in vitro* by prior inoculation with an avirulent isolate of *F. oxysporum* (AVFO) obtained from bean hypocotyl surfaces (Manning, 1983).

Therefore, based on various previous works on induced resistance or cross protection against virulent fungi with avirulent species of fungi, attempts were made to determine the possible use of avirulent *Fusarium* species such as avirulent *F. oxysporum* (AVFO) and *F. solani* as protective agents against virulent fusarial infection by *F. oxysporum* and *F. moniliforme* on asparagus plantlets, and to determine the time between induction and challenge inoculation that gives the highest levels of protection through *in vitro* test with plantlets.

3.2 Materials and Methods

Asparagus plantlets obtained through meristem tip culture were cultivated on multiplication medium for 2-3 months, and placed on filter paper slants in test tubes containing Hoagland solution (Hoagland and Arnon, 1950). Two control agar discs, or two agar discs bearing a *Fusarium* isolate, were placed in contact with roots, just below the crown. Plantlets were inoculated with an avirulent isolate of *F. oxysporum* (AVFO) or *F. solani* isolate, and then challenged with an isolate of *F. moniliforme* or a virulent *F. oxysporum* isolate at 1-, 3-, 5-, and 7-day intervals after inoculation of an avirulent *F. oxysporum* (AVFO) or *F. solani*.

3.3 Results and Discussion

3.3.1 Root Rot Disease

In the first test (Table 3.1), all different treatments showed significant differences in root rot ratings in all different interval day inoculations except in control and *F. solani* combination treatments in one and three day interval inoculations. Combination treatments of control and avirulent *F. oxysporum (AVFO)*, *F. solani* and *F. oxysporum*, and *F. solani* and *F. solani* did not show visible differences compared to control treatment. In these treatments, it has been shown that *F. solani* isolate provided better protection against *F. oxysporum* than against *F. moniliforme* in all inoculations of

Table 3.1 Results of first *in vitro* protection test with aspargus plantlets[a]

Is\Cm[d]	Root Rot Rating Means[b] Day(s) of Interval 1	3	5	7	Root Discoloration Means[c] Day(s) of Interval 1	3	5	7
CA	0.5de[e]	1.8ef	2.5cd	0.9fgh	4.0ab	4.0abcd	4.3ab	4.5abc
AA	1.8bc	3.5bcd	3.3bc	0.6fgh	4.0ab	3.8abcd	4.8ab	3.2cdef
SA	1.3cd	0.5gh	0.8efg	0.8fgh	3.5bcd	3.8abcd	4.0ab	4.3abcd
LSD[f]	1.66	0.96	0.96	0.43	1.00	0.77	1.66	1.50
CO	4.5a	4.5ab	3.5bc	3.3de	3.0bcd	3.5bcd	3.5bc	2.8efg
AO	4.3a	4.0abc	2.5cd	1.5f	3.3bcd	4.5ab	4.8ab	4.3ab
SO	0.8de	2.8de	1.3ef	1.3fg	3.3bcd	3.0cde	3.8abc	4.8ab
OO	5.0a	5.0a	5.0a	4.8ab	2.8cde	3.3bcd	3.5bc	3.5bcde
LSD[f]	1.03	1.85	1.56	1.14	1.07	1.31	1.22	1.07
CM	5.0a	5.0a	5.0a	4.8ab	4.8a	4.5ab	4.5ab	3.8abcde
AM	4.8a	3.0dc	3.3bc	2.5e	4.0ab	3.5bcd	4.8ab	5.0a
SM	5.0a	4.8ab	4.5ab	4.3abc	1.3g	2.8de	2.8c	2.5efgh
MM	5.0a	4.8ab	5.0a	5.0a	3.8bc	4.3abc	4.3ab	3.5bcde
LSD[f]	0.40	0.80	1.00	0.70	0.67	1.51	1.31	1.51
CS	0.3e	0.0h	0.3fg	0.8de	3.3bcd	4.0bcd	3.8abc	2.0fgh
AS	2.0bc	3.5bcd	4.3ab	3.8cd	4.0ab	5.0a	4.8ab	4.3abcd
SS	0.8de	1.3fg	1.8de	0.5gh	2.8cde	4.3abc	3.8abc	2.5efgh
MS	5.0a	5.0a	5.0a	5.0a	1.3g	1.0fg	1.0d	1.3h
OS	2.5b	2.5de	4.5ab	4.0bcd	2.0efg	3.3bcd	3.5bc	1.8gh
LSD[f]	1.28	1.21	1.18	1.27	0.98	0.94	0.92	1.31
COM	5.0a	5.0a	4.5ab	4.8ab	3.3bcd	4.0abcd	4.3ab	3.8abcde
AOM	5.0a	4.5ab	4.0ab	2.8e	2.5def	3.8abcd	4.5ab	4.5abc
SOM	5.0a	4.5ab	3.8abc	4.8ab	1.8efg	2.0ef	3.8abc	3.0edfg
OM[2]	5.0a	5.0a	5.0a	5.0a	3.0bcd	4.5ab	5.0a	3.3cdef
LSD[f]	0.00	0.75	0.93	0.67	1.33	0.93	1.22	1.79
CTR[g]	0.0e	0.0h	0.0g	0.0h	0.0h	0.0g	0.0d	0.0i
LSD[h]	0.9	1.1	1.1	0.8	0.9	1.0	1.0	1.2

Table 3.1 (Cont.).

Table 3.1 (Cont.).

[a]Results of each treatment were the mean of four replications. [b]Disease ratings were based on 0-5 scale where 0=no disease, and 5=death of plantlets. [c]Root discoloration ratings were based on 0-5 scale where 0=no color change, and 5=severe root discoloration. [d]Isolate combination inoculations as follows; CA=Con/Av, AA=Av/Av, SA=Fs/Av, CO=Con/Fo, AO=Av/Fo, SO=Fs/Fo, OO=Fo/Fo, CM=Con/Fm, AM=Av/Fm, SM=Fs/Fm, MM=Fm/Fm, CS=Con/Fs, AS=Av/Fs, SS=Fs/Fs, MS=Fm/Fs, OS=Fo/Fs, COM=Con/Fo+Fm, AOM=Av/Fo+Fm, SOM=Fs/Fo+Fm, OM^2=Fo+Fm/Fo+Fm, and CON=control where Con=control, Av=avirulent *F. oxysporum* (AVFO), Fs=*F. solani*, Fo=virulent *F. oxysporum*, and Fm=*F. moniliforme*. [e]Means with the same letter are not significantly different within the same interval day at P=0.05. [f]Comparison of different treatment within each group within the same interval day (LSD at P=0.05). [g]CTR=control. [h]Comparison of different treatment within the same interval day (LSD at P=0.05).

different interval days. *Fusarium solani* also reduced the mild virulence of single or double inoculations of an avirulent *F. oxysporum* isolate (AVFO) in all inoculations of different interval days. Avirulent *F. oxysporum* (AVFO) isolate did not provide much protection against *F. oxysporum* in all interval days except in seven days interval inoculation. *Fusarium solani* isolate provided much better protection against *F. oxysporum* than avirulent *F. oxysporum* (AVFO) did. However, avirulent *F. oxysporum* (AVFO) provided significant protection against virulent *F. oxysporum* at five and seven days interval inoculations. Avirulent *F. oxysporum* (AVFO) isolate provided much better protection against *F. moniliforme* in three, five and seven days interval inoculations than *F. solani* isolate did. *Fusarium solani* and *F. moniliforme* combination inoculations at all different interval days did not show any visible difference in root rot symptoms from those caused by *F. solani* and *F. moniliforme* combination inoculations. *Fusarium moniliforme* and *F. solani* combination inoculations caused more severe root rot symptoms than those caused by *F. oxysporum* and *F. solani* combination inoculations in all different interval days. Avirulent *F.* oxysporum (AVFO) and *F. solani* combination inoculations caused less severe root rot symptoms than those caused by *Fusarium moniliforme* and *F. solani* combination inoculations did. Avirulent *F. oxysporum* (AVFO), *F. oxysporum*, and *Fusarium moniliforme* combination inoculations did not show much visible difference compared to control treatment at seven days

interval inoculation, and its combination inoculation reduced root rot symptoms at five and seven days interval inoculations compared to other combination inoculations at all different interval days.

In the second test (Table 3.2), all different treatments showed significant differences in root rot ratings in all different interval day inoculations except at three, five, and seven days interval inoculations of combination of control and *F. solani*. Control and avirulent *F. oxysporum* (AVFO) combination, and dual avirulent *F. oxysporum* (AVFO) combination did not show much differences in root rot ratings, and *F. solani* and avirulent *F. oxysporum* (AVFO) combination reduced root rot symptoms highly significantly compared to control treatment. Avirulent *F. oxysporum* (AVFO) and *Fusarium oxysporum* combination treatment showed reduced root rot symptoms at one day interval inoculation, and *F. solani* provided highly significant effect in protection against *F. oxysporum* at all different interval day inoculations. *Fusarium solani* provided better protection against *Fusarium oxysporum* than avirulent *F. oxysporum* (AVFO) did at all different interval days. *Fusarium solani* failed to provide any protection against *F. moniliforme* at all different interval day inoculations, and avirulent *F. oxysporum* (AVFO) provided slight protection against *F. moniliforme* at 3 days interval inoculation. However, avirulent F. oxysporum (AVFO) did not provided sufficient protection against *F. moniliforme* at all other interval day inoculations. Combination

Table 3.2 Results of second *in vitro* protection test with asparagus plantlets[a]

Is\ Co[d]	Root Rot Rating Means[b] Day(s) of Interval 1	3	5	7	Root Discoloration Means[c] Day(s) of Interval 1	3	5	7
CA	3.0bc[e]	2.3d	2.8c	2.0c	5.0a	4.3a	2.8c	2.3cde
AA	3.5ab	3.3cd	2.3cd	2.5c	5.0a	5.0a	3.5bc	5.0a
SA	1.3de	0.5e	1.3de	1.8c	4.0a	2.8bc	4.8a	3.0bc
LSD[f]	1.50	1.12	1.29	1.66	1.41	0.87	1.38	0.96
CO	5.0a	3.8bc	4.5a	2.8bc	5.0a	4.5a	2.8c	2.5bcd
AO	3.0bc	4.5ab	4.0ab	4.0a	4.8a	4.8a	5.0a	4.8a
SO	0.5ef	3.0cd	1.5de	1.8c	4.8a	4.3a	3.8abc	3.0bc
OO	4.0ab	5.0a	5.0a	5.0a	4.5a	5.0a	5.0a	5.0a
LSD[f]	1.58	1.96	1.73	1.62	0.65	1.10	1.10	1.20
CM	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a
AM	4.5a	4.8ab	3.3bc	3.8ab	5.0a	5.0a	4.3ab	5.0a
SM	5.0a	5.0a	5.0a	5.0a	1.3c	1.3de	1.5d	1.5ef
MM	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a
LSD[f]	0.46	0.40	1.00	0.77	0.40	0.40	0.85	0.46
CS	1.3de	0.0e	0.0f	0.3d	4.5a	3.0b	3.0c	3.3b
AS	2.3cd	2.3d	4.0ab	3.8ab	2.8b	4.3a	5.0a	4.3a
SS	1.3de	3.0cd	0.8ef	0.5d	4.0a	3.3b	3.8abc	4.5a
MS	5.0a	5.0a	5.0a	5.0a	1.5c	1.0e	1.0de	1.0f
OS	2.3cd	4.5ab	4.8a	2.0c	3.0b	4.8a	2.8c	2.0de
LSD[f]	1.03	1.22	0.70	1.56	1.53	1.10	1.62	0.90
COM	5.0a	5.0a	4.5a	5.0a	5.0a	5.0a	5.0a	5.0a
AOM	5.0a	5.0a	4.5a	4.8a	5.0a	5.0a	5.0a	5.0a
SOM	5.0a	4.8ab	0.8ef	4.3a	2.8b	2.0cd	3.5bc	2.0de
OM[2]	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a	5.0a
LSD[f]	0.00	0.40	1.14	0.92	0.40	0.92	0.80	0.65
CTR[g]	0.0f	0.0e	0.0f	0.0d	0.0d	0.0f	0.0e	0.0g
LSD[h]	0.9	1.0	1.0	1.1	0.8	0.8	1.1	0.8

Table 3.2 (Cont.).

Table 3.2 (Cont.).

[a]Results of each treatment were the mean of four replications. [b]Root rot ratings were based on 0-5 scale where 0=no disease and 5=death of plantlets. [c]Root discoloration ratings were based on 0-5 scale where 0=no discoloration and 5=severe discoloration. [d]Isolate combination inoculations as follows; CA=Con/AV, AA=Av/Av, SA=Fs/Av, CO=Con/Fo, AO=Av/Fo, SO=Fs/Fo, OO=Fo/Fo, CM=Con/Fm, AM=Av/Fm, SM=Fs/Fm, MM=Fm/Fm, CS=Con/Fs, AS=Av/Fs, SS=Fs/Fs, MS=Fm/Fs, OS=Fo/Fs, COM=Con/Fo+Fm, AOM=Av/Fo+Fm, SOM=Fs/Fo+Fm, OM^2=Fo+Fm/Fo+Fm, and CON=control where Con=control, Av=avirulent *F. oxysporum* (AVFO), Fs=*F. solani*, Fo=virulent *F.oxysporum*, and Fm=*F. moniliforme*. [e]Means with the same letter are not significantly different within the same interval day at P=0.05. [f]Comparison of different treatment within each group within the same interval day (LSD at P=0.05). [g]CTR=control. [h]Comparison of different treatment within the same interval day (LSD at P=0.05).

inoculations of *F. moniliforme* and *F. solani*, and *F. oxysporum* and *F. solani* caused much more severe root rot symptoms than those of control and *F. solani* combination, avirulent *F. oxysporum* (AVFO) and *F. solani combination*, and dual combination of *F. solani* did. Inoculation combinations of *F. solani*, *F. oxysporum*, and *F. moniliforme* showed not much of a difference in root rot symptoms compared to those of control treatment. However, inoculation combinations of control, *F. oxysporum* and *Fusarium moniliforme*, avirulent *F. oxysporum* (AVFO), *F. oxysporum* and *F. moniliforme*, and dual combination of *F. oxysporum* and *F. moniliforme* showed highly significant differences in root rot symptoms compared to those of control, and 3 day interval inoculation of *F. solani*, *F. oxysporum*, and *F. moniliforme*.

3.3.2 Root Discoloration

In the first test (Table 3.1), all different treatments showed significant differences in all different interval day inoculations except in three days interval inoculation of *F. moniliforme* and *F. solani* combination. Almost all different combinations of dual or triple *Fusarium* species caused visibly and statistically significant differences compared to control treatment. Combinations of *F. moniliforme* and *F. solani* did not cause severe root discolorations at all different interval day inoculations. In one day interval inoculation test, *F. solani* and *F. moniliforme* combination, *F. oxysporum* and *F.*

solani combination, and *F. solani*, *F. oxysporum* and *F. moniliforme* combination showed less significantly different root discoloration ratings compared to control treatments. In three days interval inoculation tests, only *F. moniliforme* and *F. solani* combination, and *F. solani*, *F. oxysporum* and *F. moniliforme* combination showed less severe root discolorations. In five day interval inoculation test, only *F. moniliforme* and *F. solani* combination inoculation showed less severe root discolorations. In seven day interval inoculation, combinations of control and *F. solani*, *F. moniliforme* and *F. solani*, and *F. oxysporum* and *F. solani* caused less severe root discolorations compared to other combination treatments within the same interval day.

In the second test (Table 3.2), all treatments showed significant differences, compared to control treatment, in root discolorations in all different interval day inoculations without exception. Combinations of *F. solani* and *F. moniliforme*, and *F. moniliforme* and *F. solani* showed less severe root discoloration than other combination treatments at all different interval day treatments. *Fusarium oxysporum* and *F. solani* combination treatment showed less severe root discoloration rating in seven day interval inoculation than one, three, and seven day interval inoculation treatments. *Fusarium solani*, *F. oxysporum* and *F. oxysporum* combination inoculation treatment at three and seven days interval showed less severe root discolorations than one day and five days interval inoculation treatments did.

Based on these results it is highly possible to use avirulent fusarial species such as avirulent *F. oxysporum* (AVFO) and *F. solani* for the protection of asparagus plantlet against virulent fusarial species such as *F. oxysporum* and *F. moniliforme*. Buxton and Perry (1959) used *F. solani* to reduce pea wilt disease caused by *F. oxysporum*. In their study, they concluded that *F. solani* was a more aggressive colonizer of the root cortex which resulted in a hypersensitive reaction in the host tissue that prevented entrance by the wilt fungus *F. oxysporum*. However, in my study with asparagus plantlets, *F. solani* was not an aggressive colonizer of any tissue. Therefore, it is highly possible that some other mechanisms might be operative in protection against virulent fusarial species infections. This requires further investigation on the role of *F. solani* in protection of asparagus plantlet against virulent fusarial species infections.

Also, further study is necessary on the role of avirulent *F. oxysporum* (AVFO) in protection of asparagus plantlet against virulent fusarial species infections. Furthermore, studies to maximize the most effective combination of avirulent *F. oxysporum* (AVFO) and *F. solani* isolates to obtain maximum protection against individual *F. oxysporum* or *F. moniliforme* infection, or their combination infections are necessary. These further studies are especially essential for the management of fusarial infection on asparagus seedlings or plantlets in field or greenhouse conditions.

CHAPTER 4

COMPARISON OF PARASITIC CHARACTERISTICS OF VIRULENT AND AVIRULENT *FUSARIUM* SPECIES ON ASPARAGUS PLANTLETS

4.1 Introduction

A number of studies of the maize and *Collectotrichum graminicola* interaction have employed inoculation of the plants with conidia into a wound in the elongated internode above the brace roots (White and Humy, 1976; White *et al.*, 1987). Internode or stem segment inoculation techniques were also employed by Hooker (1957) for stalk rot caused by *Stenocarpella maydis* (Berk.) Sutton (syn. *Diplodia maydis* (Berk. Sacc.), White and Humy (1976) for stalk rot and *C. graminicola* interaction, Louis *et al.* (1988) for pepper anthracnose and *Collectotrichum capsici* interaction, and Muimba-Kankolongo and Bergstrom (1992) for work on interaction of maize anthracnose stalk rot and *C. graminicola*. These studies showed that internode or stem segment inoculation techniques were useful to determine the infectivity of conidia on wounded internodes.

Therefore, it was hoped that these techniques might be useful to determine the different infectivity of virulent and avirulent *Fusarium* species on asparagus plantlets.

4.2 Materials and Methods

4.2.1 Comparison of Conidial Population Change Among Virulent and Avirulent Fusarial Species

Three centimeter long root and stem segments were prepared from tissue cultured plantlets, and were placed on LSR medium (Desjardins *et al.*, 1987; Yang and Clore, 1973) on glass slides within sterilized petri-dishes. Inoculum was prepared by growing *Fusarium* species on PCA medium (ATTC, 1991) for 10-14 days and washing with 10 ml of sterilized distilled water. Scraped conidial suspensions were sieved through cheesecloth on top of a beaker. Out of 10^3/ml of conidial suspensions, one ul of conidial suspension was inoculated by using a micro-pipette. For an estimation of the increase of conidia in each *Fusarium* species, inoculated asparagus plantlet root and stem segments were washed with 10 ml of sterile distilled water 2 weeks after inoculation, and the number of conidia were determined by use of a hemacytometer.

4.2.2 Comparison of Disease Incidence Among Virulent and Avirulent Fusarial Species

Three centimeter long stem and root segments were prepared from tissue cultured plantlets, and were placed on LSR medium on glass slides within sterilized petri-dishes. Inoculum grown on PCA medium for 10-14 days was washed with 10

ml of sterilized distilled water. Scraped conidial suspensions were sieved through cheesecloth on top of a beaker. Out of 10^3/ml of conidial suspensions, one ul of suspension was inoculated at the bottom part of the sections by using a micro-pipette. Disease incidence or infectivity on plantlet root and stem segments was measured by using a 0-5 rating scale 2 weeks after inoculation.

4.3 Results and Discussion

4.3.1 Comparison of Conidial Population Change Among Virulent and Avirulent Fusarial Species

In the first test (Table 4.1), conidial population of avirulent *F. oxysporum* (AVFO), *Fusarium moniliforme* isolate 37, and *F. solani* did not increase significantly on root segments. However, virulent *F. oxysporum* isolate 49 showed significant increase in conidial populations on root segments compared to those of control. On stems, virulent *F. oxysporum* isolate 49 and *F. moniliforme* isolate 37 showed significant increase in conidial populations compared to those of control. *Fusarium solani* and avirulent *F. oxysporum* (AVFO) isolate did not show significant increase in conidial populations on stem segments.

In the second test (Table 4.2), the same results were obtained as shown in the first test. However, in the second test on stems, the number of conidia of avirulent *F. oxysporum* (AVFO), and *F. solani* decreased compared to those observed in

Table 4.1 Results of first conidial population change test with asparagus plantlet root and stem segments[a]

\R[b]	Root Segments (x10^3/ml)					Stem Segments (x10^3/ml)				
I[c]\	1	2	3	4	Ave[d]	1	2	3	4	Ave[d]
AV1	3.8[e]	6.8	1.0	5.6	4.3b	4.2	4.4	3.2	3.0	3.7b
FM37	2.2	0.8	2.0	1.8	1.7b	2.0	6.0	13.2	22.8	11.0a
FO49	13.0	14.6	26.2	9.8	15.9a	8.4	15.6	12.8	14.5	12.8a
FS	1.6	0.8	2.0	1.4	1.5b	4.6	5.4	2.4	2.6	3.8b
CR	0.0	0.0	0.0	0.0	0.0b	0.0	0.0	0.0	0.0	0.0b
LSD (P=0.05)					5.14					6.61

[a]Results of each replication were the mean of four observations. [b]R=replications. [c]I=isolates as follows; AV=avirulent *F. oxysporum* (AVFO), FM37=*F. moniliforme* isolate 37, FM49=virulent *F. oxysporum* isolate 49, FS=*F. solani*, and CR=control. [d]Ave=average. Means with the same letter are not significantly different at P=0.05. [e]Number of conidia divided by 10^3.

Table 4.2 Results of second conidial population change test with asparagus plantlet root and stem segments[a]

\R[b]	Root Segments (x10^3/ml)					Stem Segments (x10^3/ml)				
I[c]\	1	2	3	4	Ave[d]	1	2	3	4	Ave[d]
AV	3.0[e]	4.0	7.4	10.6	6.0b	1.8	1.8	1.8	2.0	1.9b
FM37	4.8	1.4	0.8	4.0	2.8b	0.6	4.4	16.6	25.6	11.8a
FO49	21.8	22.8	31.6	12.2	22.1a	18.4	12.4	21.4	19.0	17.8a
FS	2.2	1.0	2.0	2.8	2.0b	5.8	2.0	3.2	2.2	3.3b
CR	0.0	0.0	0.0	0.0	0.0b	0.0	0.0	0.0	0.0	0.0b
LSD (P=0.05)					6.00					8.23

[a]Results of each replication were the mean of four observations. [b]R=replications. [c]I=isolates as follows; AV=avirulent *F. oxysporum* (AVFO), FM37=*F. moniliforme* isolate 37, FO49=virulent *F. oxysporum* isolate 49, FS=*F. solani*, and CR=control. [d]Ave=average. Means with the same letter are not significantly different at P=0.05. [e]Number of conidia divided by 10^3.

the first test. The number of conidia of all isolates increased slightly in all tests except in tests on stems with avirulent *F. oxysporum* (AVFO) and *F. solani* infections.

4.3.2 Comparison of Disease Incidence Among Virulent and Avirulent Fusarial Species

In the first test (Table 4.3), all *Fusarium* species showed significant differences in both root and stem rots compared to those of control. However, avirulent *F. oxysporum* (AVFO) isolate and *F. solani* isolate showed low level of disease incidence on both root and stem segments. *Fusarium oxysporum* isolate 49 showed rather severe disease incidence on both root and stem segments, and *F. moniliforme* caused highly severe rots on both root and stem segments. Avirulent *F. oxysporum* (AVFO) isolate caused less severe rots on stem segments than those on root segments, and the same result was observed with *F. solani* isolate. *Fusarium moniliforme* isolate 37 caused highly severe rots on both root and stem segments. Virulent *F. oxysporum* isolate 49 caused slightly more severe rots on stem segments than on root segments. However, there was no significant difference between the two.

In the second test (Table 4.4) with roots, all isolates of *Fusarium* species showed significant differences compared to control treatment. However, isolates of *F. oxysporum* and *F. moniliforme* caused highly severe rot symptoms, and avirulent *F. oxysporum* (AVFO) and *F. solani* isolates caused less visible

Table 4.3 Results of first disease incidence test with asparagus plantlet root and stem segments[a]

\R[b]	Root Segment Rot Rating Means[c]									Stem Segment Rot Rating Means[c]								
I[d]\	1	2	3	4	5	6	7	8	Ave[e]	1	2	3	4	5	6	7	8	Ave[e]
AV	1	1	1	5	3	1	1	4	2.1c	1	3	1	1	2	1	2	1	1.5c
FM 37	5	5	5	5	5	5	5	5	5.0a	5	5	5	5	5	5	5	5	5.0a
FO 49	3	4	4	2	3	3	3	4	3.3b	4	5	4	4	3	3	3	5	3.9b
FS	0	3	4	3	3	3	2	2	2.5bc	0	0	0	0	0	2	3	1	0.8d
CR	0	0	0	0	0	0	0	0	0.0d	0	0	0	0	0	0	0	0	0.0e
LSD (P=0.05)									0.98									0.74

[a]Results of each replication were the mean of three observations. [b]R=replications. [c]Disease ratings were based on 0-5 scale where 0=no rot symptoms, and 5=severe rot symptoms. [d]I=isolates as follows; AV= avirulent *F. oxysporum* (AVFO), FM37=*F. moniliforme* isolate 37, FO49=virulent *F. oxysporum* isolate 49, FS =*F. solani*, and CR=control. [e]Ave=average. Means with the same letter are not significantly different at P=0.05.

Table 4.4 Results of second disease incidence test with asparagus plantlet root and stem segments[a]

	Root Segment Rot Rating Means[c]									Stem Segment Rot Rating Means[c]								
I[d]\R[b]	1	2	3	4	5	6	7	8	Ave[e]	1	2	3	4	5	6	7	8	Ave[e]
AV	4	4	3	5	1	3	1	0	2.6b	3	4	2	3	0	0	0	1	1.6b
FM 37	5	5	5	5	3	5	5	5	4.8a	5	5	5	5	5	4	5	5	4.9a
FO 49	5	5	5	5	3	5	5	5	4.8a	4	5	4	5	5	4	5	5	4.6a
FS	1	0	1	3	2	2	1	2	1.5c	0	0	2	2	0	0	2	0	0.8bc
CR	0	0	0	0	0	0	0	0	0.0d	0	0	0	0	0	0	0	0	0.0c
LSD (P=0.05)									0.91									0.91

[a]Results of each replication were the mean of three observations. [b] R=replications. [c]Disease ratings were based on 0-5 scale where 0=no rot symptoms, and 5=severe rot symptoms. [d]I=isolates as follows; AV=avirulent *F. oxysporum* (AVFO), FM37 =*F. moniliforme* isolate 37, FO=virulent *F. oxysporum* isolate 49, FS=*F. solani*, and CR=control. [e]Ave=average. Means with the same letter are not significantly different at P=0.05.

rots. On stem segments, isolates of *F. oxysporum* and *F. moniliforme* caused the same severe rots as shown in a test on root segments. *Fusarium moniliforme* isolate 37 caused slightly more severe rots on stem segments than on root segments even if there was no significant difference between the two. Avirulent *F. oxysporum* (AVFO) isolate showed less severe rot symptoms on stem segments with no significant difference than it did on root segments. *Fusarium solani* isolate showed reduced symptom development on stem segments with significant difference compared to that on root segments.

Overall, *Fusarium moniliforme* isolates showed higher disease incidence than virulent *F. oxysporum* isolates did without statistically significant difference between the two. Isolates of *F. moniliforme* showed higher disease incidence on stem segments than on root segments without significant statistical difference. There was no visible difference on symptoms on root and stem segments caused by *F. oxysporum* isolate, and there was no statistically significant difference, either. Isolates of avirulent *F. oxysporum* (AVFO) and *F. solani* showed lower disease incidence on stem segments than on root segments. *Fusarium solani* isolate showed statistical difference between disease incidence on stem segments and that on root segments.

If higher increased number of conidial population is directly related to higher disease incidence, these results support previous work by Lacy (1977) and Johnston *et al.* (1979). They showed *F. oxysporum* was isolated from discolored

vascular root tissue and cortical root lesions, while *Fusarium moniliforme* was primarily isolated from cortical stem and crown lesions. *Fusarium moniliforme* was found to be the main causal agent of stem and crown rot disease while *F. oxysporum* was found to be the causal agent of root rot and wilt symptoms. Similar observations were reported previously by Damicone (1980), Gilbertson (1981), Manning (1983), Damicone and Manning (1985), LaMondia and Elmer (1988), and Lee and Manning (1992).

CHAPTER 5

HISTOPATHOLOGICAL STUDY OF VIRULENT AND AVIRULENT FUSARIA INFECTING ASPARAGUS PLANTLETS

5.1 Introduction

There have been many studies of penetration, ingress, and subsequent colonization and systemic distribution of *Fusarium oxysporum* within the roots of susceptible host plants such as cotton (*Gossypium* sp.) (Khadr and Snyder, 1967), flax (*Linum* sp.) (Tisdale, 1959), pea (*Pisum sativum*) (Hepple, 1963; Nyvall and Haglund, 1976), alfalfa (*Medicago sativa*) (Chi *et al.*, 1964), red clover (*Trifolium pratense*) (Chi *et al.*, 1964), banana (*Musa* sp.) (Rishbeth, 1955; Sequeira *et al.*, 1958; Stover, 1957; Wardlaw, 1930a; Wardlaw, 1930b; Wardlaw, 1931), cabbage (*Brassica* sp.) (Anderson and Walker, 1935; Smith and Walker, 1930), carnation (*Dianthus caryophyllus*) (Pennypecker and Nelson, 1972), sweet potato (*Ipomoea batatas*) (McClure, 1949), asparagus (*Asparagus officinalis* L.) (Smith and Peterson, 1983; Smith and Peterson, 1985) and onion (*Allium* sp.) (Abawi and Lorbeer, 1971). The means by which *F. oxysporum* penetrates roots and then enters vascular elements differs. In some cases, penetration is direct, while in others wounds are required.

The most common sites of direct penetration are located at or near the root tip of both tap roots and lateral roots (Pennypacker, 1981). The fungus commonly penetrates root hairs or epidermal cells near the root cap, just behind the root tip, or within the zone of elongation. It then moves intercellularly and intracellularly through the root parenchyma tissue, enters the primary meristem, and invades the differentiating tracheary elements of the protostele.

Cortical decay fusaria are principally confined to the cortex of their host. Fungal growth in the tissue may be intercellular, intracellular, or a combination of both and is frequently facilitated by enzymatic degradation of the middle lamella and cell walls. Once the cortex is colonized, the fungus sporulates externally by means of sporodochia formed through the stromata and forms chlamydospores in the necrotic cells of the cortex. The pathogens may or may not be confined to the cortex by the endodermis and when they are not, vascular penetration occurs during the late stages of the infection (Pennypecker, 1981).

The host responds to infection in many cases by the production of hypertrophied, hyperplastic cells resembling a periderm. This response occasionally limits the spread of the pathogen. Graham (1955) reported delineation of the lesion area by a periderm-like layer of cortical cells. These cells appeared to play a part in restricting *F. oxysporum* var. *redolens* to the cortex of asparagus. Similar results for cortical rot fusaria were reported (Arnett and Witcher, 1974;

Cunningham, 1953; Pierre *et al.*, 1970). Lack of any host response leads to the complete maceration and collapse of the cortical cells, vascular systems, and eventual death of the plant or plantlets (Pennypacker, 1981).

Within the zone of differentiation, and more mature areas of the root, the endodermal cells and pericycle are penetrated inter- or intracellularly. Protoxylem vessels are entered through thin vessel walls between spiral thickenings. The vessel elements are then invaded with subsequent fungal spread from vessel to vessel through pits in the vessel wall. The number of infection sites, age of the plant, length of time the pathogen is in contact with the root surface, and inoculum density all influence symptom development (MacHardy and Beckman, 1981).

The continued development and spread of *F. oxysporum* within a susceptible host has been studied in greatest detail in *Fusarium* wilt of tomato (*Lycopersicon esculentum*) (Chambers and Cordon, 1963; Elgersma *et al.*, 1972; Phillips *et al.*, 1967; Scheffer and Walker, 1954) and banana (Beckman and Halmos, 1962; Beckman *et al.*, 1962; Trujillow, 1963; Beckman *et al.*, 1972). In tomato, hyphae of *F. oxysporum* f. sp. *lycopersici* are confined to protoxylem and metaxylem vessels in the lower nodes during the early stages of infection. Within the hypocotyl, lateral spread from vessel to vessel through pits soon results in extensive invasion of secondary as well as primary xylem elements. There follows a massive stage of colonization in which an enormous number of spores

are produced, followed by a rapid and extensive invasion of the primary and secondary xylem elements within the upper stem and petioles. The pathogen moves forward within the xylem elements in a manner similar to that in banana roots as described below. At later stages, the pathogen invades and proliferates in xylem parenchyma.

In banana, following entrance into the xylem elements of adventitious roots, *F. oxysporum* f. sp. *cubense* spreads into the rhizome stele and then invades the tracheary elements of the pseudostem. Extensive colonization of rhizome xylem occurs prior to invasion of the pseudostem and is analogous to the extensive colonization of the hypocotyl when susceptible tomato and cabbage plants are attacked. Microconidia are the principal agents that cause upward distribution of *Fusarium* through sap stream until they encounter an end wall or perforation plate. Germinated microconidia then penetrate porous barriers and sporulate above the obstruction, and *Fusarium* becomes distributed quickly along xylem vessels. Initial movement through the root xylem may be slow in young roots, but in older, mature metaxylem vessels, the pathogen can advance in surges of 30 cm with every new generation of spores (MacHardy and Beckman, 1981).

Occlusion of vessel elements is apparently a common mechanism by which many plants limit vascular invasion (Beckman, 1964). The occluding mechanism was operative in banana against various soil microorganisms and formae specialis of *F. oxysporum* isolated from other hosts. Sweet

potato, broccoli (*Brassica oleracea*), passion flower (*Passiflora* sp.), squash (*Cucurbita* sp), and sesame (*Sesamum indicum*) all had localized infection within six or seven days after root inoculations with either a mixture of micro-organisms or *F. oxysporum* f. sp. *lycopersici* (Beckman, 1966). The resistance of cultivars to their respective *F. oxysporum* formae specialis has been correlated with rapid vascular occlusion by gels in cotton (Bugbee, 1970), by tyloses in tomato (Beckman *et al.*, 1972), and by gel gums, tyloses, and vascular collapse in banana (Wardlaw, 1930a; Beckman and Halmos, 1962; Beckman *et al.*, 1962; Beckman *et al.*, 1972). Gel formation is apparently a general phenomenon common to resistant-type responses (Vander Molen *et al.*, 1977).

Susceptibility was correlated with delayed occlusion in cotton (Bugbee, 1970) and tomato (Beckman *et al.*, 1972) and gel breakdown and delayed tylosis in banana (Beckman *et al.*, 1962). In banana, the susceptibility of the cultivar Gros Michel to *F. oxysporum* f. sp. *cubense* at 28°C was correlated with delayed development of physical barriers and distribution and build-up of the pathogen. At 21°C or 30°C the localization of infections by gels and tyloses and the absence of symptoms was comparable to that in a resistant banana cultivar (Beckman *et al.*, 1962).

Tomato cultivars susceptible to *F. oxysporum* f. sp. *lycopersici* had resistance mechanisms that were effective against this pathogen if these mechanisms were first made operative by pre-inoculation (3 days in advance) with the

nonpathogen, *Cephalosporium* (Langton, 1969; Phillips *et al.*, 1967). The induced resistance was associated with occlusion of the primary xylem vessels by tyloses that occurred immediately above the infection sites. The occlusions reduced the spread of the pathogen from primary to secondary xylem, from the roots to the hypocotyl, and from the hypocotyl to the first internode (Pennypecker and Nelson, 1972).

Vascular wilt pathogen *Fusarium oxysporum* f. sp. *asparagi* infection on primary roots of asparagus was reported by Smith and Peterson (1983 and 1985). They studied the structural changes during the prepenetration and penetration stages of infection. The meristematic regions appeared to be the primary infection site as evidenced by the aggregation of fungus in that region. Hyphae of *Fusarium* invaded the root cap by both inter- and intracellular pathways. Penetration of the epidermis was inter- and intracellular; if intercellular, the fungus became intracellular within this layer and followed this route through the cortex. They did not find specialized infection structures. Meristematic cells frequently developed wall appositions which appeared to act as a barrier to fungal ingress. Necrosis occurred in and around penetrated cells and sometimes occurred considerably in advance of the fungal front (Smith and Peterson, 1983). Smith and Peterson (1985) found meristematic cells were the first barrier to fungal advance. They also found that colonized cells and uninfected cells contiguous with infected cells showed many cytological changes.

In asparagus, it is generally known that *Fusarium* species cause primarily cortical decay and stem-girdling, and eventual death of plants. Therefore, based on previously published reports, attempts were made to study the infection process of virulent and avirulent fusarial species within a short period of time and over extended periods, and to study interactions of virulent and avirulent fusarial species in the protection of asparagus plantlet against virulent *Fusarium* species with avirulent *Fusarium* species.

5.2 Materials and Methods

Plantlets were obtained from tissue cultures of meristem and internodes of asparagus female clone, NJ362M. Asparagus plantlets, grown for two to three months in multiplication medium after root formation in rooting medium, were transplanted into a modified sterile glass-board unit (Figure 5.1) (Kendall and Leath, 1974) or into a sterile 15 cm diameter plastic petri dish moist chamber apparatus. Glass-board units were washed and autoclaved before each use, and 15 cm diameter plastic petri dishes were sterilized with 70% alcohol. Inoculum was prepared by growing fusarial species on PCA and washing with distilled sterile water.

For a study on the infection process of virulent and avirulent fusarial species within a short period time, several designated parts of roots and stems in each plantlet were inoculated with one aliquot (1 ul) of each different *Fusarium*

Figure 5.1 Modified glass-board unit apparatus.

Several designated parts of stems and roots in each plantlet (<—) were inoculated with one aliquot (1 ul) of *Fusarium* inoculum. Transplanted asparagus plantlets were acclimatized for one day before inoculation of fusarial species. Modified glass-board unit apparatus was substituted, as necessary, with improvised 15-cm diameter plastic petri dish moist chamber apparatus which has the same components as the modified glass-board unit. Filter paper and Hoagland solutions were autoclaved prior to experiment. Glass units were autoclaved, and plastic units were washed with 70% alcohol before each use. A: glass-board covered with filter paper saturated with Hoagland solution, B: Hoagland solution reservoir to provide nutrients and moisture within glass-board unit, C: inoculation points (<—), D: asparagus plantlet, E: glass lid (or plastic petri dish cover), F: large glass moist chamber (250x80 mm) or 15-cm diameter plastic petri dish, and G: small glass moist chamber (130x40 mm) or 9-cm diameter plastic petri dish for support of glass-board.

inoculum (Figure 5.1) one day after the plantlets were transplanted. Evaluation and sample selection for microscopic observation were conducted at 1, 3, 5, and 7 days.

For a study on the infection process of virulent and avirulent fusarial species over extended periods, several designated parts of stems and roots in each plantlet were inoculated with one aliquot (1 ul) of each different *Fusarium* inoculum (Figure 5.1) one day after the plantlets were transplanted. Evaluation and sample collection for microscopic observation were conducted over time within four weeks.

For a study of interactions of virulent and avirulent fusarial species in asparagus plantlet protection against virulent *Fusarium* species with avirulent *Fusarium* species, one aliquot (1 ul) of virulent *F. oxysporum* or *F. moniliforme* was inoculated 1, 3, 5, or 7 days after the initial inoculation of avirulent *F. oxysporum* (AVFO) or *F. solani* conidial inoculum. Evaluation and sample selection for microscopic observation were conducted four weeks after challenge inoculations.

For studies on simultaneous infections of virulent and avirulent fusarial species within a short period and over extended periods, and for study on interactions of virulent and avirulent fusarial species under simultaneous inoculation conditions, one aliquot (1 ul) of each different fusarial inoculum ($3x10^3$/ml), pre-stained with different fluorescent stains, was inoculated, in dual or in combinations, at several designated parts of roots and stems of plantlet.

Two or three non-inoculated control roots and stems were maintained on each plantlet for all different tests. Selected root and stem pieces, inoculated with virulent and/or avirulent *Fusarium* species in all different tests, were collected and cut with sterile razor blades, and fixed in FAA or AFA fixative (Schneider, 1981). Selected materials were sectioned longitudinally or transversely by hand, mounted on slides, and stained with aniline blue W. S. (Schneider, 1981). In cases where paraffin embedding technique (Butler, 1979) was used, 10 um thin paraffin ribbon sections were prepared followed by staining with aniline blue W. S. The selected stained sections from each treatment in each experiment were examined with light microscopy. In cases where the fluorescent dyes such as calcofluore, DAPI, or Hoechst 33258 and 33342 were used as pre- or post-stain, staining procedures and observations under UV light fluorescent microscopy were performed according to previous descriptions (Butt *et al.*, 1989; Cohen *et al.*, 1987; Gantotti and Woodske, 1991; Panwar *et al.*, 1987; Schans *et al.*, 1982; Smith and McCully, 1978).

5.3 Results and Discussion

5.3.1 Study of Infection Processes of Virulent and Avirulent Fusarial Species Within a Short Period

All *Fusarium* species infect asparagus plantlets through primary and lateral root tips (Figure 5.2), natural wounds

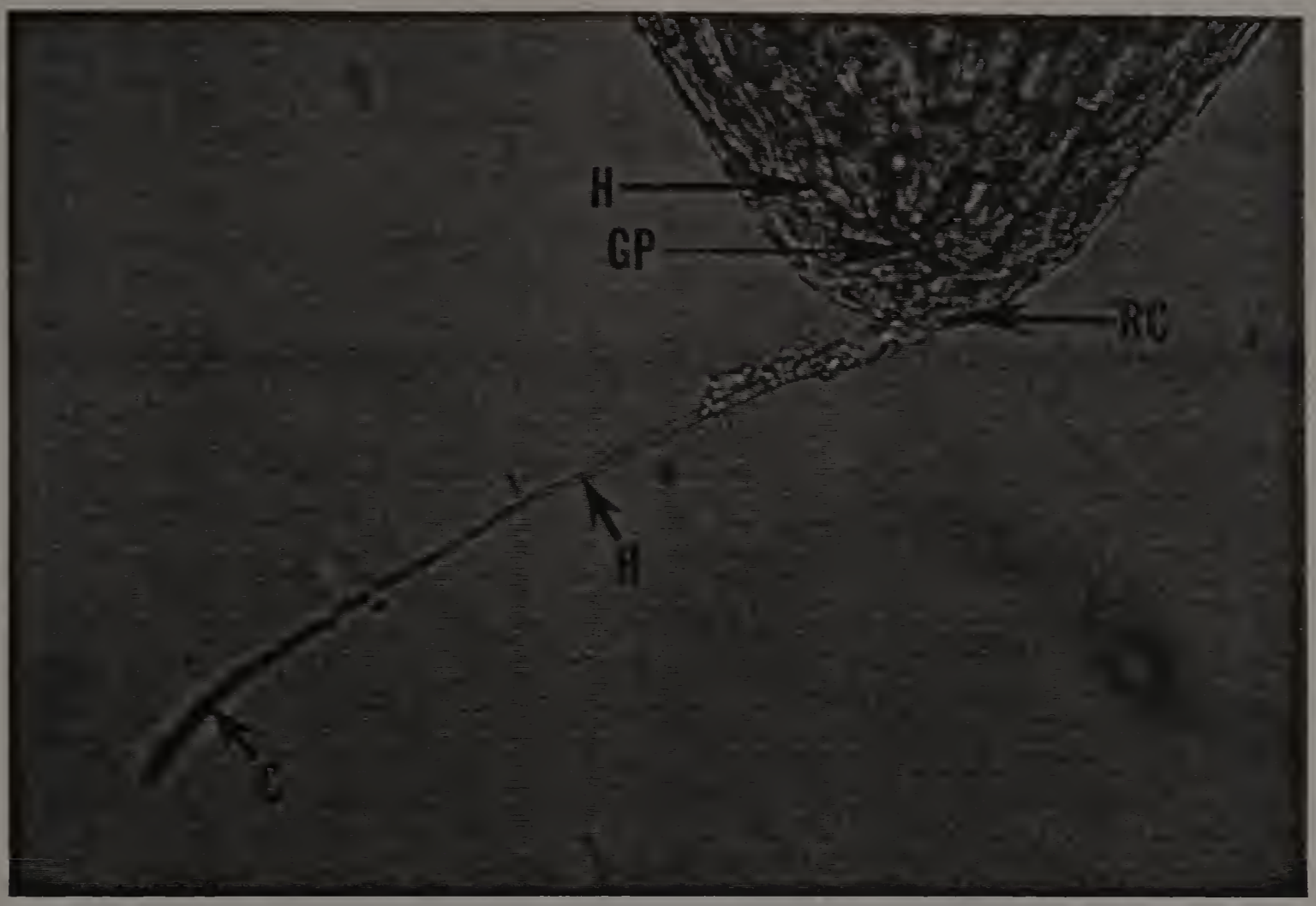

Figure 5.2 ***Fusarium moniliforme*** **infection on root tip of asparagus plantlet 24 hours after inoculation (x395).**

Note hyphae growing toward, and on root tip area. Root pieces were cut longitudinally by hand, mounted on slides, stained with aniline blue W. S., and observed under light microscopy. C: conidia, GP: growing point, H: hyphae, and RC: root cap.

(Figure 5.3), and between the walls of the epidermal cells directly. Some penetration was appressorium-like and direct (Figure 5.4) and I assume that the penetration was taking place between the walls of the epidermal cells, not directly through the outer walls unless there was a damaged tissue area. This same observations were reported previously by Graham (1955) in asparagus study. I also assume that the meristematic region of the root can act as a major infection site because of heavy mycelial aggregation in that region as reported in previous asparagus study (Graham, 1955; Smith and Peterson, 1983 and 1985). However, there were differences among virulent and avirulent fusarial species in their speed of infection on asparagus plantlet root and stem tissues.

Virulent fusarial species such as *Fusarium moniliforme* and *F. oxysporum* isolates penetrated tissues very quickly, and they infected the tissues faster than avirulent fusarial species such as avirulent *F. oxysporum* (AVFO) and *F. solani* isolates did. *Fusarium moniliforme* infected root tissues slightly faster than *F. oxysporum* did, not only within a twenty-four-hour period but also during the whole infection process. Hyphae of *F. moniliforme* grew more abundantly inside and outside of the epidermal and cortical tissues of asparagus plantlets than *F. oxysporum* did during the whole infection processes. Where *Fusarium moniliforme* was infecting root tip areas, the hyphae were concentrated in the area of the root cap and along the epidermal area immediately basipetal to the root cap forming a heavy thallus network around the apex. The

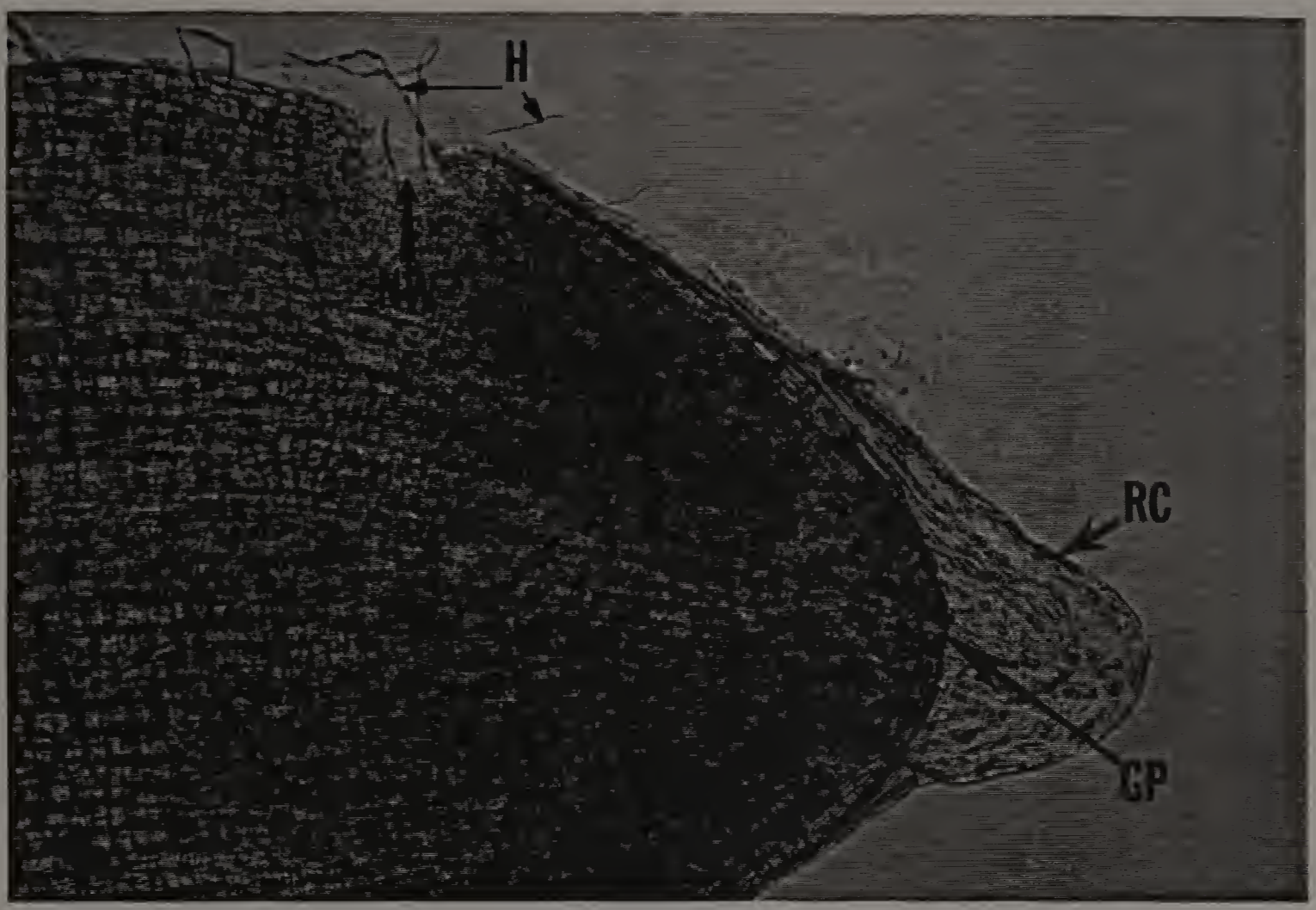

Figure 5.3 Fusarial infection on naturally wounded primary root of asparagus plantlet (x395).

Root pieces were cut longitudinally by hand, mounted on slides, stained with aniline blue W. S., and observed under light microscopy. GP: growing point, H: hyphae, NW: natural wound, and RC: root cap.

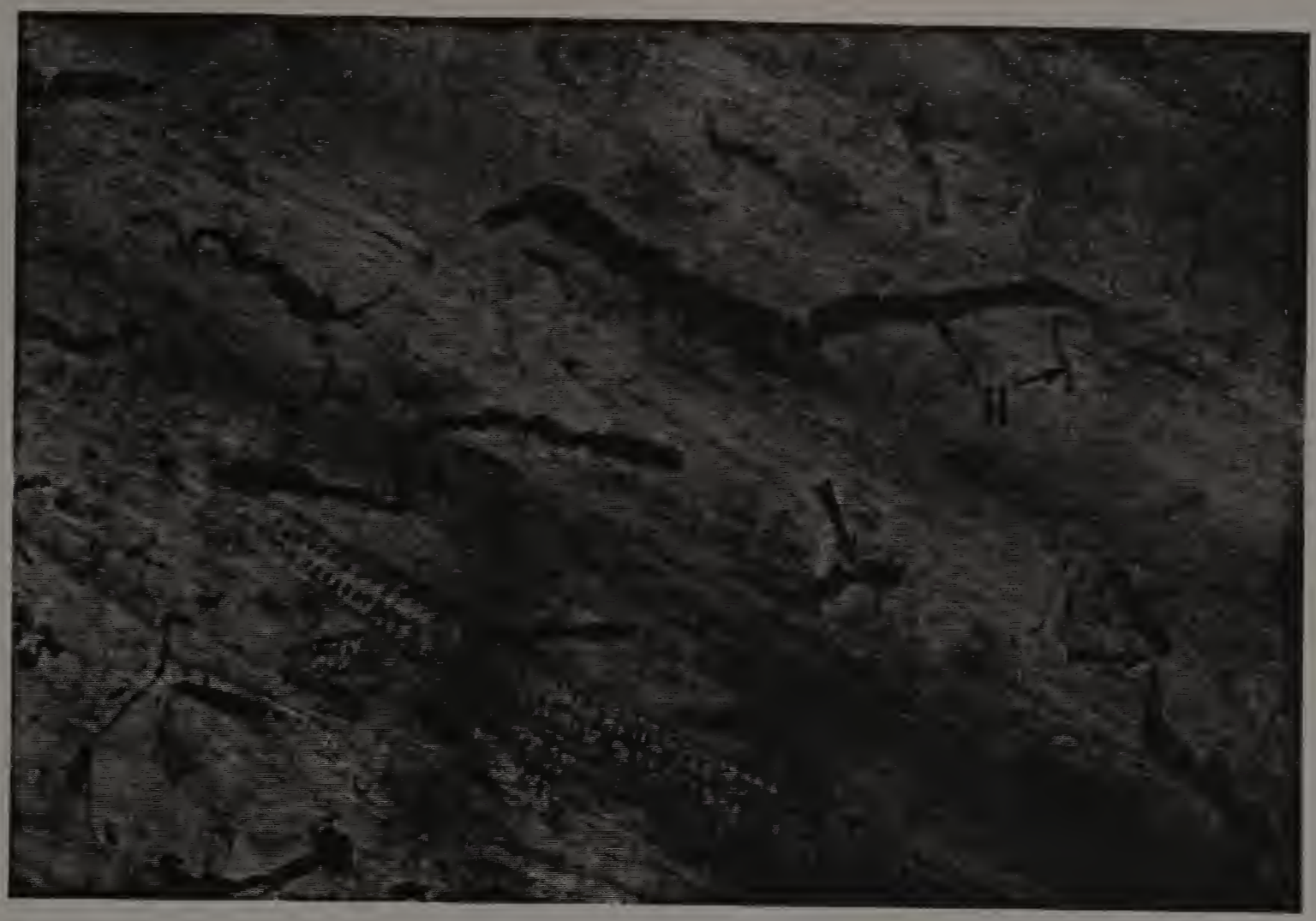

Figure 5.4 ***Fusarium oxysporum*** **infection on asparagus plantlet root 24 hours after inoculation (x370).**

Note appressorium-like structures (<——). Root pieces were cut longitudinally by hand, mounted on slides, stained with aniline blue W. S., and observed under light microscopy. H: hyphae, and XY: xylem.

same phenomenon was observed in inoculations of *F. oxysporum* in root tissues. Where *F. moniliforme* was infecting stem tissues, heavy hyphal growth on the surface of asparagus plantlet stems was also observed. Hyphae of *F. oxysporum* grew more abundantly inside the tissues than outside of the tissues. These aggregation of fungus in the infecting regions was reported by Graham (1955), and Smith and Peterson (1983 and 1985) in their studies with asparagus plants. Hyphae of *Fusarium oxysporum* grew mainly intracellular in root tissues. Hyphal growth and infection processes of avirulent *F. oxysporum* (AVFO) were similar to those of virulent isolates of *Fusarium oxysporum* except the fact that avirulent *F. oxysporum* (AVFO) accumulated more on the epidermal area than virulent *Fusarium oxysporum* isolate did over a longer period. No fungal infection was observed within twenty four hours of inoculation of *F. solani* isolate, and penetration was observed about 2 days after inoculation. Hyphae of *F. solani* grew very slowly over time, and mostly remained to the epidermal and cortical areas in early stages of infection. This delayed penetration of *F. solani* is in contrast to the report by Buxton and Perry (1959) who reported that *F. solani* was aggressive colonizer of the root cortex of pea and reduced the pea wilt disease by *F. oxysporum*. However, results I have obtained support previous observations by Chi *et al*. (1964).

During and after the initial infection of virulent *Fusarium* species, there were clear signs of symptom development in primary and lateral root tips, and epidermal

tissues of roots and stems. In areas where symptoms were weakly expressed, there was a presence of small brownish elliptical regions of infection. Dark brown lesions were visible especially in root tip areas and epidermal tissues of stems near crown regions. These lesions seemed to represent a primary stage in the development of the symptoms. Some isolates of virulent *Fusarium* species also caused lesions near the points of origin of the lateral roots.

Examination of roots and stems revealed masses of fungal hyphae in the cortical tissue (Figure 5.5). At this early stage of disease development, hyphae were developed externally from epidermal and cortical cells of asparagus plantlets in some occasions, and this result supports previous observations by Bennett (1928), Dahl (1934), and Pugh *et al*. (1933).

Virulent *Fusarium* species, such as *F. oxysporum* and *F. moniliforme*, produced hyphae more abundantly than avirulent *Fusarium* species, such as avirulent *F. oxysporum* (AVFO) and *F. solani* did. As the disease progressed, hyphae penetrated into both intracellular (Figures 5.6 and 5.7) and intercellular (Figure 5.7) spaces. Some of the *F. moniliforme* and *F. oxysporum* hyphae were coiled just inside or outside of the cells before penetration into nearby cells. Some isolates of *F. moniliforme* and *F. oxysporum* formed coils within the plantlet cells just after penetration (Figure 5.8). Hyphae were located in the peripheral areas between the cells, and the hyphae extended for long distances in longitudinal sections of stems (Figure 5.9). Occasionally, lateral hyphae

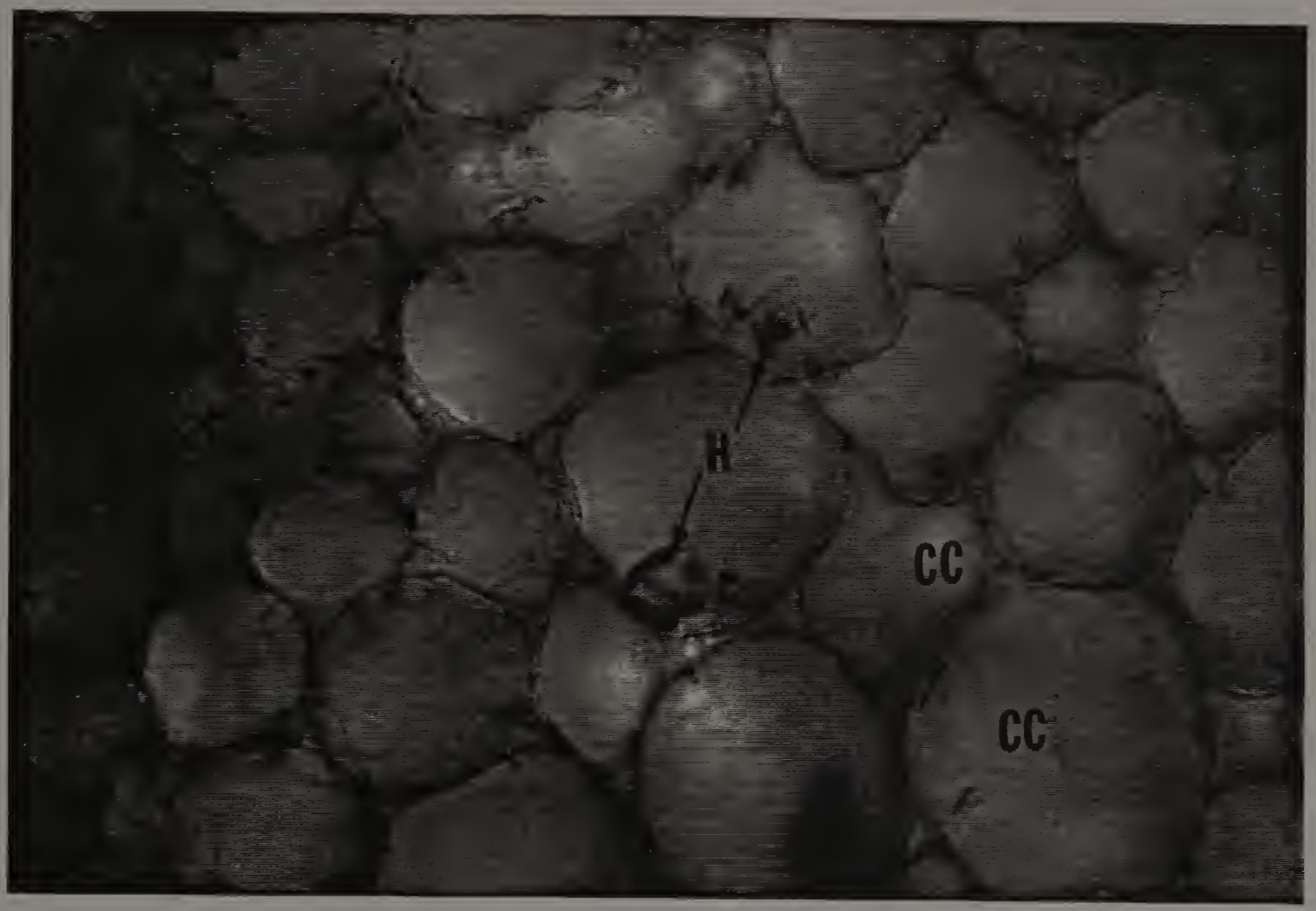

Figure 5.5 Avirulent *F. oxysporum* (AVFO) infection on cortical cells of asparagus plantlet stem seven days after inoculation (x980).

Note hyphal masses within cortical cells. Stem pieces were cut longitudinally by hand, mounted on slides, stained with aniline blue W. S., and observed under light microscopy. CC: cortical cells, and H: hyphae.

Figure 5.6 Avirulent *F. oxysporum* (AVFO) infection on asparagus plantlet root epidermal area 24 hours after inoculation (x995).

Note hyphae within the plantlet cells. Stem pieces were cut longitudinally by hand, mounted on slides, stained with aniline blue W. S., and observed under light microscopy. CW: cross wall, H: hyphae, HS: hyphal swelling, and S: septa of hyphae.

Figure 5.7 ***Fusarium moniliforme*** **infection on stem of asparagus plantlet 24 hours after inoculation (x956).**

Note the hyphae growing intercellularly (<—) and intracellularly (<<—). Stem pieces were cut longitudinally by hand, mounted on slides, stained with aniline blue W. S., and observed under light microscopy. ED: endodermal area, H: hyphae, HT: hyphal tip, and S: septa.

Figure 5.8 Avirulent *F. oxysporum* (AVFO) infection on epidermal area of asparagus plantlet root seven days after inoculation (x1004).

Note hyphal coils (MMC) within a cell after penetration. Root pieces were cut longitudinally by hand, mounted on slides, stained with aniline blue W. S., and observed under light microscopy. CW: cell walls, H: hyphae, HT: hyphal tip, and MMC: mycelial mats coiled.

Figure 5.9 ***Fusarium oxysporum*** **infection on stem of asparagus plantlet five to seven days after inoculation (x780).**

Note hyphae growing upward for long distances. Stem pieces were cut longitudinally by hand, mounted on slides, stained with aniline blue W. S., and observed under light microscopy. H: hyphae, and TR: tracheary element.

were found entering still intact cortical cells by means of a constriction and padlike swellings or appressorium-like structures (Figure 5.10).

Infected and stunted cells take a deeper stain with aniline blue W. S. and lactophenol (Schneider, 1981), presumably because of the greater permeability of their walls. However, infected areas of tissues were stained less clearly with calcofluore stains, probably because of loss of vitality in those infected areas of tissues. After the hyphae penetrate, they were generally intercellular. However, at later stages, they may become more intracellular almost immediately. In the fully developed areas of root and stem tissues, they extend, both intercellularly and intracellularly, in lateral and vertical directions. The hyphae were variable in their manner of growth within the tissues of the asparagus root and stem and on the surface of root and stem, and form numerous small swellings as they progress through the cortex. These swellings can be regarded as appressoria because their formation apparently precedes the passage of an extremely small intercellular space, or the penetration of a cell wall. The colonization of the host asparagus plantlet extends to the parenchyma and vessel elements (Figures 5.11 and 5.12).

For a study on simultaneous infections of virulent and avirulent fusarial species within a short period, it was not possible to differentiate hyphae of different fusarial species within the plantlet tissues due to differences in reflection

Figure 5.10 *Fusarium moniliforme* infection on stem of asparagus plantlet seven days after inoculation (x1011).

Note hyphae entering intact cortical cells by means of a constriction (<—) and pad-like swelling or a appressorium-like structure (<<—). Stem pieces were cut longitudinally by hand, mounted on slides, stained with aniline blue W. S., and observed under light microscopy. H: hyphae, and HT: hyphal tip showing pad-like swelling or appressorium-like structure.

Figure 5.11 ***Fusarium moniliforme*** **infection on stem of asparagus plantlet two weeks after inoculation (x556).**

Note hyphal growth near or within vessel elements. Stem pieces were cut in cross section by hand, mounted on slides, stained with aniline blue W. S., and observed under light microscopy. CC: Cortical cells, H: hyphae, and V: vessel elements.

Figure 5.12 ***Fusarium moniliforme*** **infection on root tissue of asparagus plantlet three weeks after inoculation (x997).**

Note hypha growing near xylem. Root pieces were cut longitudinally by hand, mounted on slides, stained with calcofluore white, and observed under light microscopy. CC: plantlet cortical cells, H: hyphae, HT: hyphal tip, S: septa, and XY: xylem.

of lights by different fluorescent stains used for each different fusarial species. Also, autofluorescence of the plantlet tissues, and insufficient destaining of plantlet tissues made it difficult to differentiate hyphae from plantlet tissues.

In conclusion, all fusarial species infected asparagus plantlets through primary and lateral root tips, natural wounds, and between walls of the epidermal tissues directly. Some penetration was appressorium-like. Virulent fusarial species penetrated tissue very quickly and infected the tissues faster than avirulent fusarial species did. *Fusarium moniliforme* grew abundantly within and without the plantlet tissues, and *F. oxysporum* grew more abundantly within the cortical and epidermal tissues than outside of the epidermal and cortical tissues. Avirulent *F. oxysporum* (AVFO) showed similar growth patterns as virulent *F. oxysporum* did within a short period of growth stage. *Fusarium solani* did not show any growth within twenty-four-hour period, started to grow within two days after inoculation. Most of fusarial infections in this early stage were intercellular and confined to cortical regions. This result supports previous reports by Christou and Snyder (1962), Hancock (1968), and Sparnicht and Roncadori (1972). Some grew outward from epidermal and cortical tissue regions. At later stages of infection, some species started to cause more intracellular infections as reported previously (Adams, 1921; Pugh et al., 1933; Hadley, 1973; Phillips, 1962).

5.3.2 Study of Infection Processes of Virulent and Avirulent Fusarial Species Over Extended Periods

Avirulent *F. oxysporum* (AVFO) did not cause any damage in xylem and parenchyma cell regions, and there were heavy accumulation of mycelial mats within and around the epidermis compared to control treatment (Figures 5.13 and 5.14). This epidermal area was heavily stained with aniline blue W. S. In a root tip infection, avirulent *F. oxysporum* (AVFO) caused minor damage over time, and, in some cases, the root cap was dissolved by infection (Figure 5.15). However, there were heavy mycelial mats accumulated on and around the epidermal areas of the root tip (Figure 5.15).

In a *Fusarium solani* infection, there was no extensive damage on cells (Figure 5.16) except in limited areas of the epidermal region (Figure 5.17). *Fusarium solani* infection caused minor damages on the epidermal regions even if it promoted lateral root formation (Figure 5.17). Also, *Fusarium solani* increased surface area of plantlet primary and lateral roots (Figure 5.17). Therefore, it was clear that *F. solani* reduced asparagus plantlet root rot and wilt diseases by promoting lateral root formation, and, increasing the root surface areas. This promoted lateral root formation and increased surface area of primary and lateral roots might have

Figure 5.13 Control treatment in a test to study infection process of different fusarial species over extended periods (x98).

Note intact xylem, parenchyma cells, and epidermal areas. Also, note lateral root growing. Root pieces were embedded in paraffin, sectioned thin (10 um), stained with aniline blue W. S., and observed under light microscopy. EN: endodermal area, EP: epidermal area, LR: lateral root, N: nuclei, PA:parenchyma and VA: vascular system.

Figure 5.14 Avirulent *F. oxysporum* (AVFO) infected root tissues of asparagus plantlet four weeks after inoculation (x94).

Note heavily stained areas within and around the epidermal area, intact tracheary element, and intact parenchyma cells. Root pieces were embedded in paraffin, sectioned thin (10 um), and stained with aniline blue W. S., and observed under light microscopy. ED: endodermal area, EP: epidermis, N: nuclei, PA: parenchyma, PP: paraffin particles, and TR: tracheary element.

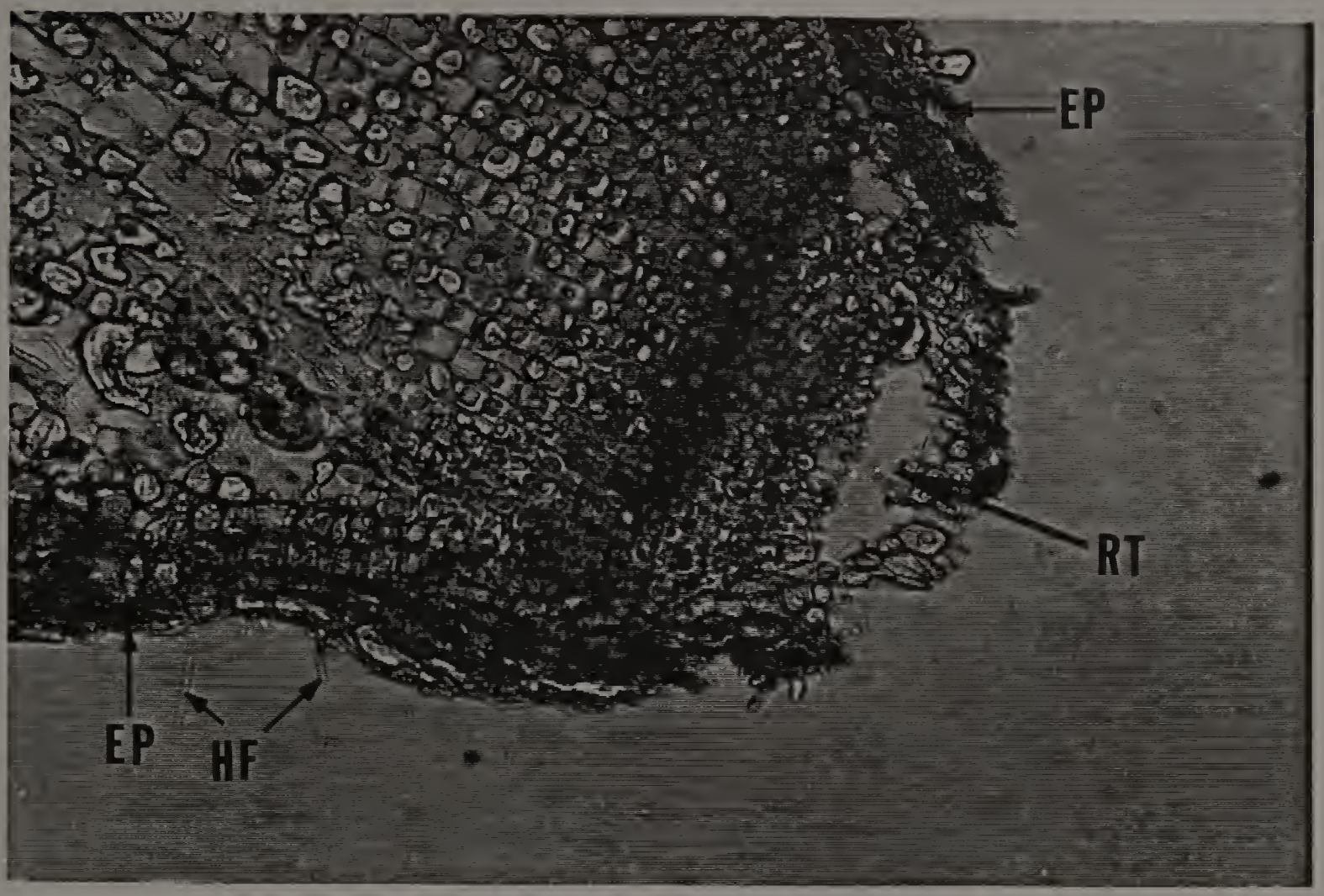

Figure 5.15 Avirulent *F. oxysporum* (AVFO) infected root tip of asparagus plantlet four weeks after inoculation (x94).

Note root cap area dissolved by infection. Epidermal areas of the root tip were heavily stained. In this heavily stained epidermal area, heavy mycelial mat accumulations were observed. Root pieces were embedded in paraffin, sectioned thin (10 um), stained with aniline blue W. S., and observed under light microscopy. EP: epidermal area, HF: hyphal fragments, and RT: root tip area destroyed.

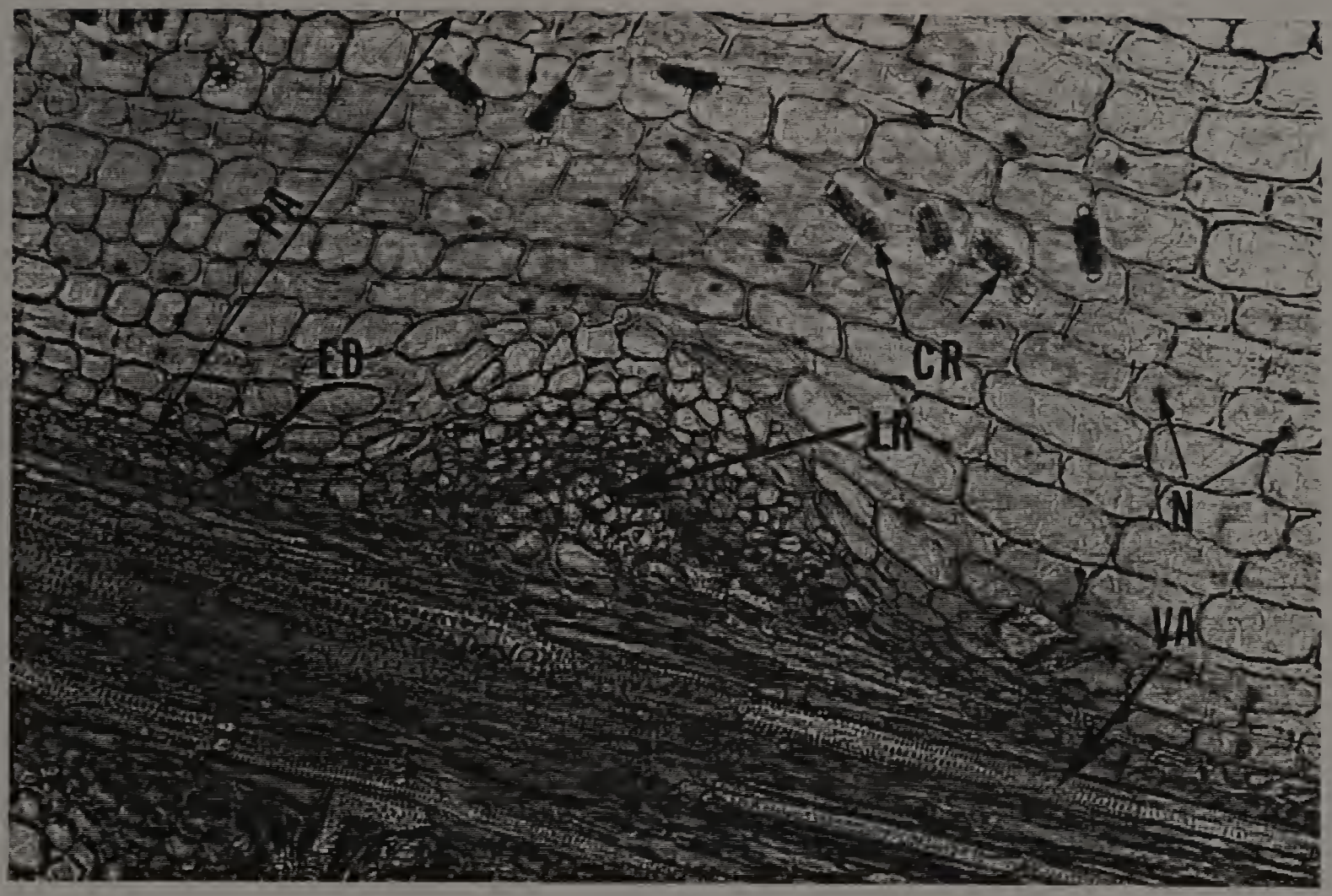

Figure 5.16 ***Fusarium solani*** **infected root tissues of asparagus plantlet four weeks after inoculation (x98).**

Note intact parenchyma cells, vascular system, and endodermis. Lateral roots are growing vigorously as indicated by increased cells near lateral root growing regions. Root pieces were embedded in paraffin, sectioned thin (10 um), stained with aniline blue W. S., and observed under light microscopy. CR: crystals, ED: endodermis, LR: lateral roots growing, N: nuclei, PA: parenchyma, and VA: vascular system.

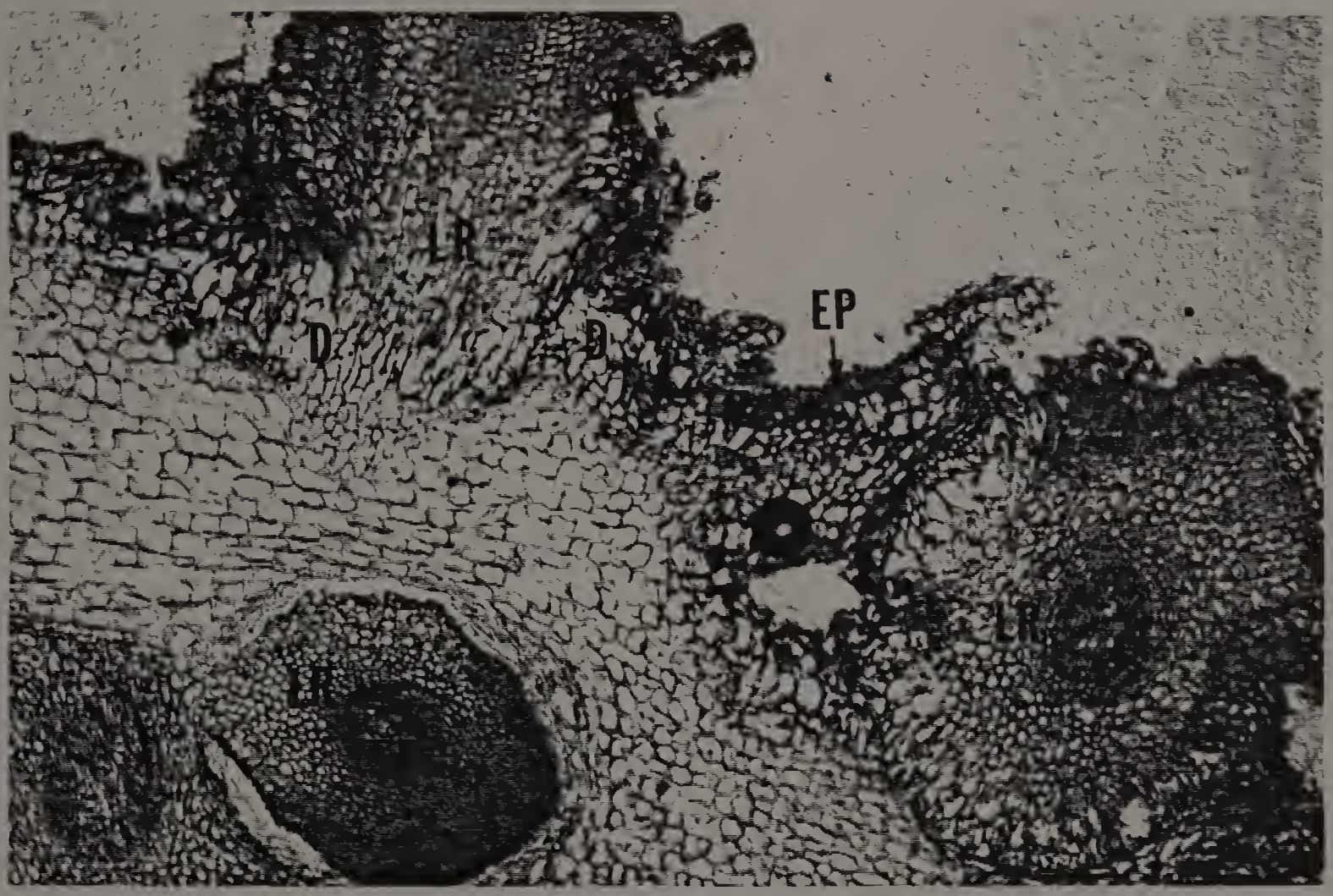

Figure 5.17 ***Fusarium solani*** **infected root tissues of asparagus plantlet four weeks after inoculation (x37).**

Note proliferation of lateral roots, and minor damages on epidermal region. Also, note the increased surface area of plantlet root. Root pieces were embedded in paraffin, sectioned thin (10 um), stained with aniline blue W. S., and observed under light microscopy. EP: epidermis, D: damaged area, and LR: lateral root.

caused by undefined toxins produced by *F. solani*, or by mechanisms similar to those observed in mycorrhizal fungi.

In contrast to avirulent *F. oxysporum* (AVFO) and *F. solani* infections, however, virulent *F. oxysporum* and *F. moniliforme* caused extensive damages on plantlet tissues (Figures 5.18, 5.19, 5.20, and 5.21). *Fusarium oxysporum* and *F. moniliforme* both caused heavy damages on vessel elements (Figures 5.18 and 5.20) and epidermal regions (Figures 5.19 and 5.20). Highly virulent *F. moniliforme* infection caused quite extensive damage on plantlet tissues, and dissolved the tissues inside the epidermal areas completely (Figure 5.21). These observations support the facts that *F. oxysporum* and *F. moniliforme* cause cortical rots in the early stage as described in previous section and vascular system destruction over longer periods (Green, 1981).

For a study on simultaneous infections of virulent and avirulent fusarial species over extended periods, it was not possible to differentiate hyphae of different fusarial species within the plantlet tissues due to differences in reflections of lights by different fluorescent stains used for each different fusarial species. Also, auto-fluorescence of the plantlet tissues, and insufficient destaining of plantlet tissues made it difficult to differentiate hyphae from plantlet tissues.

In conclusion, at the later stages of infection, virulent fusarial species such as *F. oxysporum* and *F. moniliforme* caused heavy damage in epidermal, cortical, and xylem tissues.

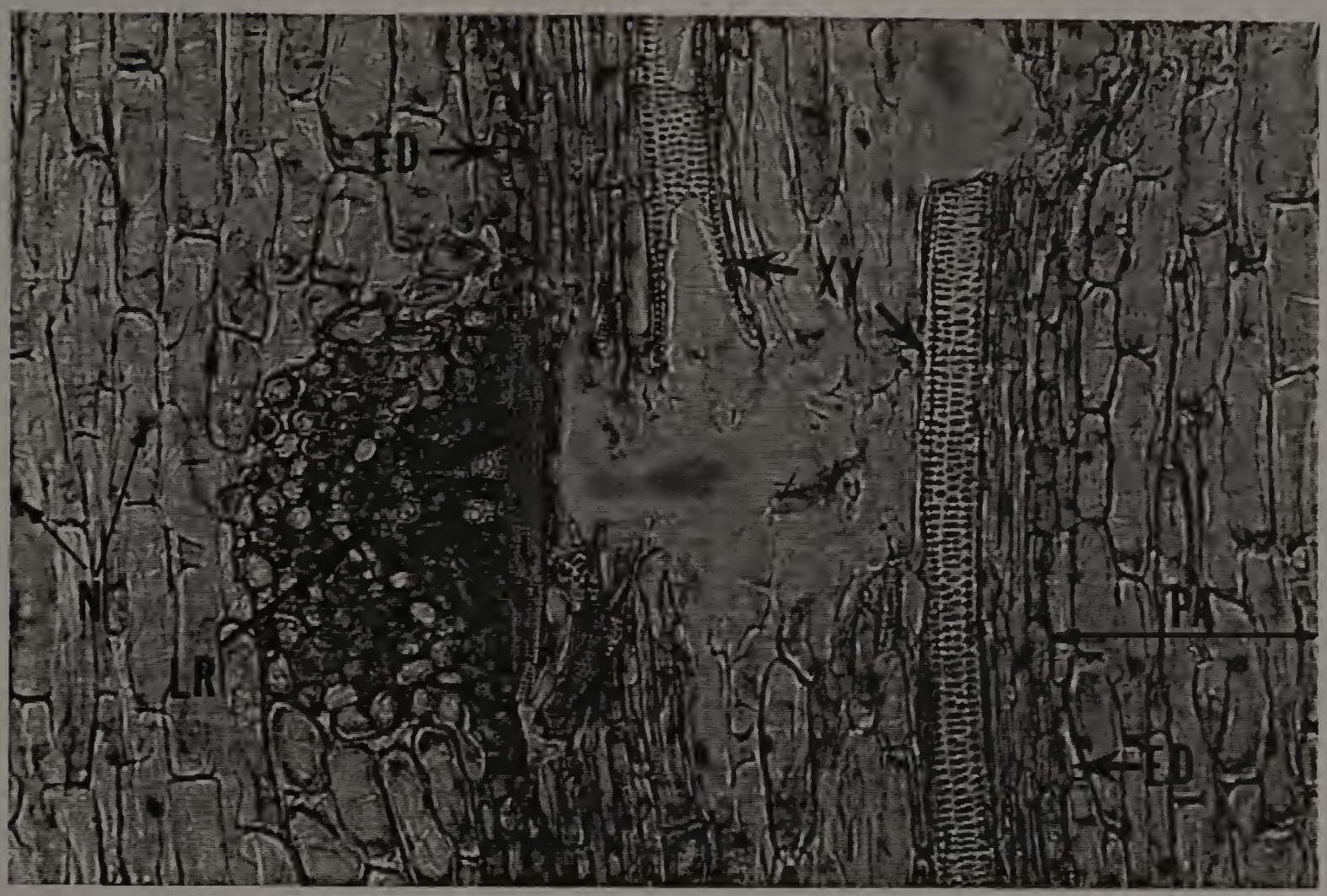

Figure 5.18 ***Fusarium oxysporum*** **infected root tissues of asparagus plantlet four weeks after inoculation (x122).**

Note extensive damages on xylem, and endodermal regions by infection. Root pieces were embedded in paraffin, sectioned thin (10 um), stained with aniline blue W. S., and observed under light microscopy. ED: endodermis, LR: lateral root, N: nuclei, PA: parenchyma, and XY: xylem.

Figure 5.19 ***Fusarium oxysporum*** **infected tissues of asparagus plantlet root four weeks after inoculation (x42).**

Note extensive damages on epidermis, and cortical cells near epidermal region. Root pieces were embedded in paraffin, sectioned thin (10 um), stained with aniline blue W. S., and observed under light microscopy. EP: epidermal area, and LR: lateral root.

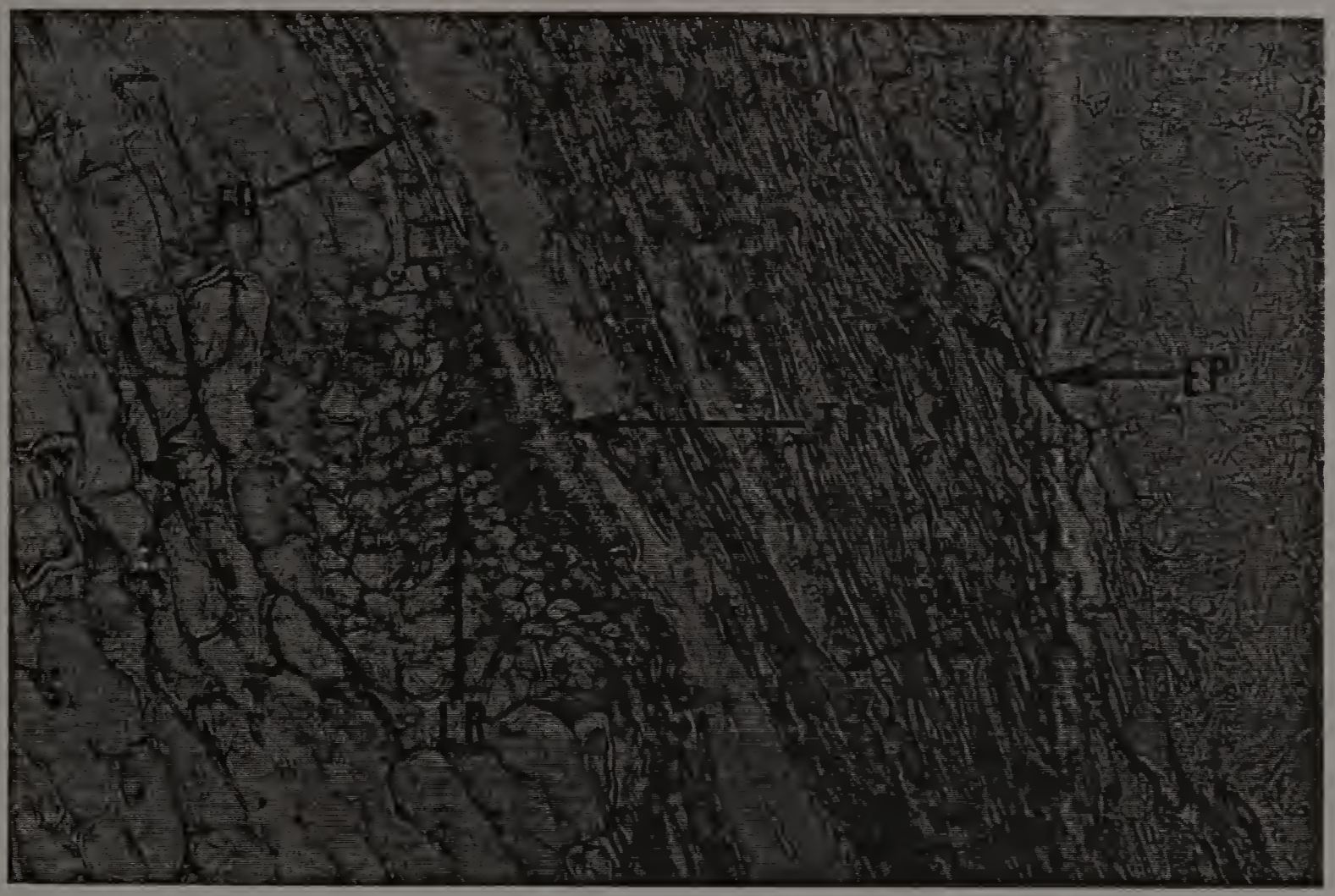

Figure 5.20 ***Fusarium moniliforme*** **infected root tissues of asparagus plantlet four weeks after inoculation (x95).**

Note extensive damages in tracheary elements, endodermis, epidermal regions, parenchyma cells, and outer edges of lateral root in early stage of development. Root pieces were embedded in paraffin, sectioned thin (10 um), stained with aniline blue W. S., and observed under light microscopy. ED: endodermis, EP: epidermal area, LR: lateral root, PA: parenchyma, and TR: tracheary element.

Figure 5.21 ***Fusarium moniliforme*** **infected stem tissues of asparagus plantlet four weeks after inoculation (x38).**

Note total destruction of tissues inside the epidermal areas. Root pieces were embedded in paraffin, sectioned thin (10 um), stained with aniline blue W. S., and observed under light microscopy. EP: epidermis, IT: internal tissues destroyed, and LR: lateral root.

Highly virulent *F. moniliforme* infection dissolved the cell regions inside the epidermal areas completely. Avirulent *F. oxysporum* (AVFO) isolate did not cause any major damage on plantlet tissues except minor damage in root tip areas. Hyphae of avirulent *F. oxysporum* (AVFO) isolate accumulated heavily in epidermal regions as evidenced by heavy staining with aniline blue W. S. *Fusarium solani* infection did not cause any damage on plantlet tissues except in limited areas of the epidermal region. *Fusarium solani* promoted lateral root formation, and increased surface area of primary and lateral roots.

5.3.3 Study of Interactions of Virulent and Avirulent Fusarial Species in Asparagus Plantlet Protection Against Virulent Fusarial Species with Avirulent Fusarial Species

In a combination inoculation of *F. solani* and avirulent *F. oxysporum* (AVFO) in which avirulent *F. oxysporum* (AVFO) was inoculated 5 days after *F. solani* was inoculated, no heavy damage on plantlet cells was observed (Figure 5.22). This result shows protection of asparagus plantlets against mild infection of avirulent *F. oxysporum* (AVFO) with prior inoculation of *F. solani*, and this supports protection test result described in Chapter 3. Also, the number of lateral roots increased, and heavy accumulations of mycelial mats on epidermal areas were observed. It was assumed that the

Figure 5.22 Asparagus plantlet root tissues protected with *Fusarium solani* against avirulent *F. oxysporum* (AVFO) (x98).
Note almost intact cortical and epidermal cells, and heavily stained epidermal areas. Root pieces were embedded in paraffin, sectioned thin (10 um), stained with aniline blue W. S., and observed under microscopy. ED: endodermis, EP:epidermis, LR: lateral root, N: nuclei, PA: parenchyma, and TR: tracheary element.

increased number of lateral roots was caused by *F. solani,* and heavy accumulations of mycelial mats on epidermal regions were assumed to be caused by avirulent *F. oxysporum* (AVFO) based on the information obtained from individual isolate inoculation test described in previous section.

Avirulent *F. oxysporum* (AVFO) provided slightly better protection against *F. moniliforme* than it did against virulent *F. oxysporum* (Figures 5.23 and 5.24). In a combination of avirulent *F. oxysporum* (AVFO) and *F. moniliforme* inoculation where avirulent *F. oxysporum* (AVFO) was used a protection agent, there was no extensive damage within the vicinity of epidermal areas, and there was heavy damage near tracheary elements and endodermal areas (Figure 5.23). However, this combination inoculation caused less damage compared to avirulent *F. oxysporum* (AVFO) and *F. oxysporum* combination inoculation where avirulent *F. oxysporum* (AVFO) was used as a protection agent (Figure 5.24). In this avirulent *F. oxysporum* (AVFO) and *F. oxysporum* inoculation, not only vessel elements and parenchyma cells but also epidermal areas were damaged heavily (Figure 5.24). Therefore, it was clear that avirulent *F. oxysporum* (AVFO) formed mechanical barriers on the epidermal areas, and thus provided protection against virulent fusarial infections. However, in other studies with avirulent *F. oxysporum* as a biocontrol agent for celery (*Apium graveolens* L. var. *dulce* (Miller) Pers.) (Schneider, 1984), cucumber (Paulitz *et al.*, 1987) and *tomato* (Louter and Edgington, 1990), they explained the mechanisms involved as

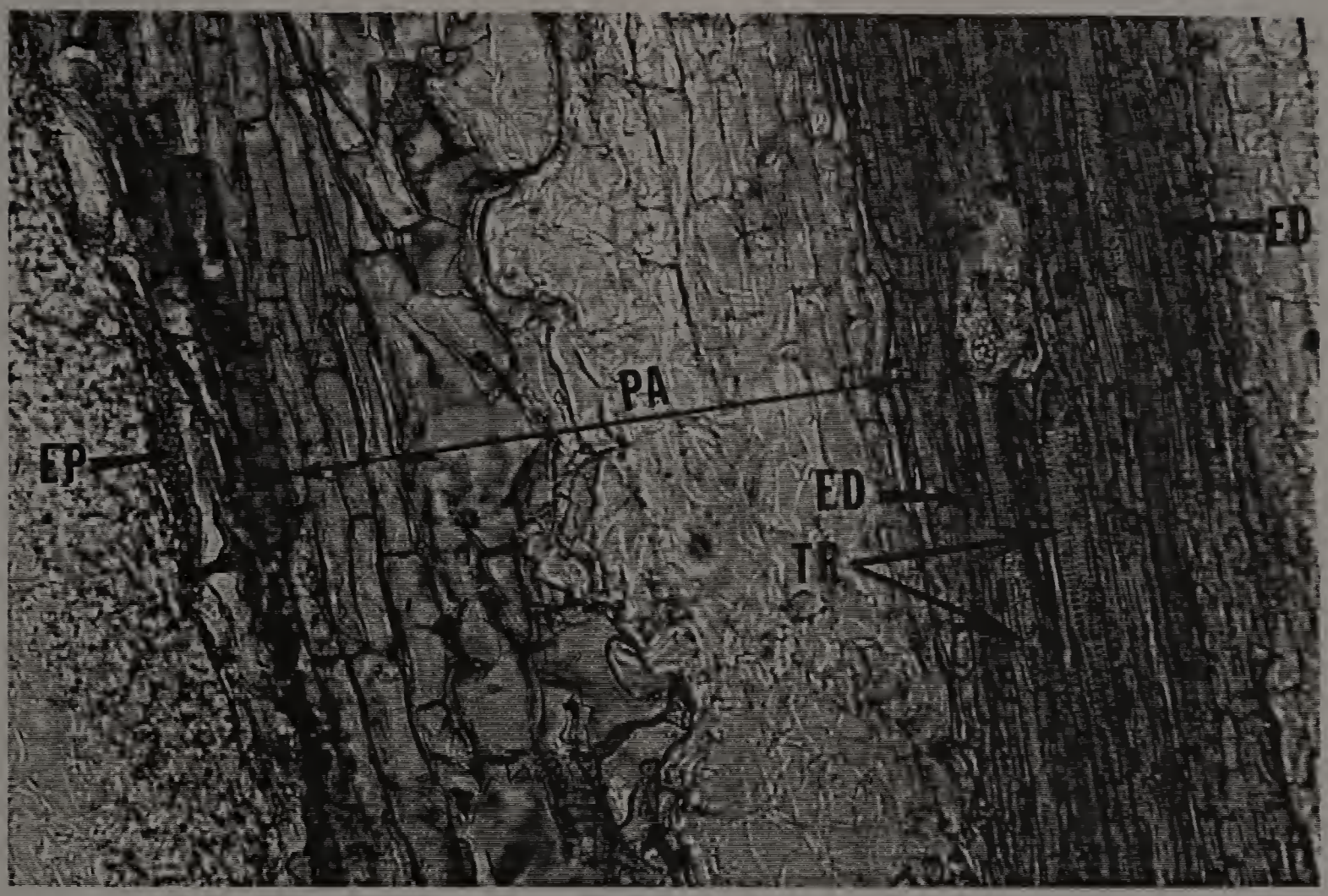

Figure 5.23 Asparagus plantlet root tissues protected with avirulent *F. oxysporum* (AVFO) against *F. moniliforme* infection (x98).

Note almost intact epidermal region, partially damaged parenchyma, and intact endodermal areas. Root pieces were embedded in paraffin, sectioned thin (10 um), stained with aniline blue W. S., and observed under light microscopy. ED: endodermis, EP: epidermal areas, PA: parenchyma, and TR: tracheary element.

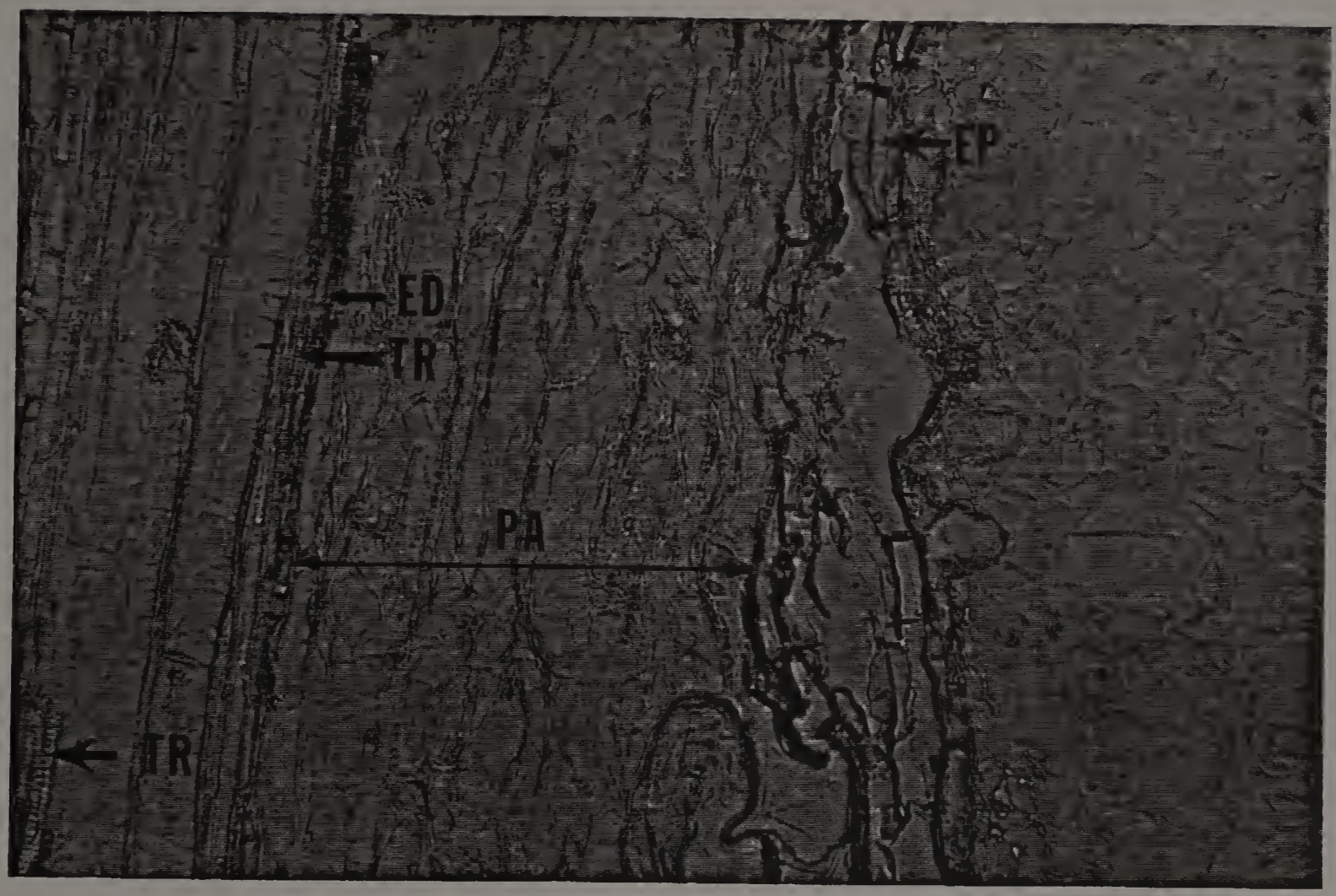

Figure 5.24 Asparagus plantlet root tissues protected with avirulent *F. oxysporum* (AVFO) against virulent *F. oxysporum* (x98).

Note heavy damages in tracheary elements, parenchyma cells, and epidermal areas. Root pieces were embedded in paraffin, sectioned thin (10 um), stained with aniline blue W. S., and observed under light microscopy. ED: endodermal area, EP: epidermal area, PA: parenchyma, and TR: tracheary elements.

competition for nutrients or space between avirulent and virulent isolates of *Fusarium* species. There are many reports of use of avirulent or weakly virulent isolates of *F. oxysporum* or formae speciales for the control of each different host plants, and they explained the mechanisms involved as competition for nutrients or space, or antagonisms among virulent and avirulent (or weakly virulent) isolates (Davis, 1967 and 1968; Langton, 1969; Mars and Molot, 1977; Gessler and Kuc, 1982; Ogawa and Komada, 1984).

Fusarium solani provided much better protection against *F. oxysporum* than it did against *F. moniliforme* (Figures 5.25 and 5.26). In a combination of *F. solani* and *F. oxysporum* inoculation where *F. solani* was used as a protection agent, there was no extensive damage in parenchyma cell regions even if there were minor damages on epidermal areas and vessel elements (Figure 5.25). Compared to this combination inoculation, there was overall heavy damage of plantlet cells in a combination inoculation of *F. solani* and *F. moniliforme* where *F. solani* was used as a protection agent. In this *F. solani* and *F. moniliforme* combination inoculation, there was damage in tracheary elements and tissues inside the epidermal areas. In this combination inoculation, tissues inside the epidermal areas were totally dissolved with few cells left (Figure 5.26). Therefore, it was clear that *F. solani* reduced asparagus plantlet root rot and wilt diseases by promoting lateral root formation and increasing the root surface areas. This result is different from previous reports on the use of

Figure 5.25 Asparagus plantlet root tissues protected with *F. solani* against *F. oxysporum* (x35).

Note intact parenchyma cell regions, and minor damages in epidermal areas and vessel elements. Root pieces were embedded in paraffin, sectioned thin (10 um), stained with aniline blue W. S., and observed under light microscopy. ED: endodermis, EP: epidermal area, LR: lateral root, N: nuclei, PA: parenchyma, and TR: tracheary element.

Figure 5.26 Asparagus plantlet root tissues protected with *F. solani* against *F. moniliforme* (x34).

Note overall heavy damage in tracheary element, and tissues inside the epidermis. Also, tissues inside the epidermal areas were totally dissolved with few cells left. Root pieces were embedded in paraffin, sectioned thin (10 um), stained with aniline blue W. S., and observed under light microscopy. EP: epidermis, IT: internal tissues destroyed, and TR: tracheary element.

F. solani as a biocontrol agent (Bega, 1954; Buxton and Perry, 1959; Louter and Edgington, 1990; Magie, 1980; McClure, 1951). Bega (1954) and McClure (1959) protected sweet potato from wilt caused by *Fusarium* with pre-inoculations with *F. solani*. Buxton and Perry (1959) used *F. solani* to reduce pea wilt disease caused by *F. oxysporum*. In their study, Buxton and Perry (1959) concluded that *F. solani* caused a hypersensitive reaction in the host tissue that prevented entrance by the wilt fungus *F.oxysporum*. Magie (1980) obtained reduction of *Fusarium* yellows and corm rot of gladiolus by pre-inoculation of avirulent *F. solani* in field plot test, and he indicated possible antagonistic interactions between avirulent *F. solani* and *F. oxysporum* f. sp. *gladioli*. Louter and Edgington (1990) explained the mechanism involved in reduction of symptoms in tomato by avirulent *F. solani* as competition for nutrients or space between avirulent and virulent isolates of *Fusarium* species.

For a study on interactions of virulent and avirulent fusarial species under simultaneous inoculation conditions, it was not possible to differentiate hyphae of different fusarial species within the plantlet tissues due to differences in reflections of lights by different fluorescent stains used for each different fusarial species. Also, autofluorescence of the plantlet tissues, and insufficient destaining of plantlet tissues made it difficult to differentiate hyphae from plantlet tissues.

In conclusion, in a combination of avirulent *F. oxysporum* (AVFO) and *F. moniliforme* inoculation where avirulent *Fusarium oxysporum* (AVFO) was used as a protection agent, there was no extensive heavy damage within the vicinity of epidermal areas, but there was heavy damage near tracheary elements and endodermal areas. In a combination inoculation of avirulent *F. oxysporum* (AVFO) and *F. oxysporum* where avirulent *F. oxysporum* (AVFO) was used as a protection agent, there was heavy damage not only in vessel elements and parenchyma cells but also in epidermal areas. *Fusarium solani* and *F. oxysporum* combination inoculation where *F. solani* was used as a protection agent caused no extensive damage in parenchyma cells even if there were minor damages on epidermal regions and vessel elements. *Fusarium solani* and *F. moniliforme* combination where *F. solani* was used as a protection agent caused overall heavy damage in tracheary elements and tissues inside the epidermal areas. This combination totally dissolved tissues inside the epidermal areas.

CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

Protection tests and histopathological studies showed that avirulent *F. oxysporum* (AVFO) and *F. solani* can be used as protection agents for asparagus plantlets against infection by virulent fusaria, such as *F. oxysporum* and *F. moniliforme*.

Protection of asparagus plantlets was obtained through pre-inoculations with avirulent *F. oxysporum* (AVFO) or *F. solani*. Where protection was obtained, five or seven days of interval between protection and challenge inoculations was required. Avirulent *F. oxysporum* (AVFO) provided better protection against *F. moniliforme* infection than against *F. oxysporum* infection. However, *F. solani* provided better protection against *F. oxysporum* infection than against *F. moniliforme* infection.

Histopathological studies showed that avirulent *F. oxysporum* (AVFO) provided protection in asparagus plantlets by accumulation of hyphae on or around the epidermal area, thus forming barriers against virulent fusarial infections. Similar results were reported previously (Graham, 1955; Arnett and Witcher, 1974; Cunningham, 1953; Pierre et al., 1970). However, these previous reports deal mainly with periderm-like layer of cortical cells formed in reaction to fungal infections, instead of accumulation of hyphae on epidermal areas. *Fusarium solani* provided protection of asparagus

plantlets by increasing lateral roots and increasing surface area of primary and secondary roots of asparagus plantlets. These results are different from a previous report by Buxton and Perry (1959) who explained the role of *F. solani* as causing a hypersensitive reaction in host tissue, in the reduction of pea wilt disease caused by *F. oxysporum*. These results are the first report of avirulent *F. oxysporum* (AVFO), isolated from bean hypocotyl (Manning, 1983), and *F. solani*, isolated from asparagus plants grown in the field, as protection agents on tissue cultured asparagus plantlets against virulent *F. oxysporum* and *F. moniliforme* isolate infections. Also, these results are the first report that elucidate the mechanical mechanisms of protection of avirulent *F. oxysporum* (AVFO) and *F. solani* against virulent *F. oxysporum* and *F. moniliforme* infections on asparagus plantlets.

Based on the results mentioned in previous chapters, further research is necessary to completely understand the mechanism of protection and the interactions among different fusarial species during infection of asparagus plantlets. Suggested research areas include:

1. Use of different clonal asparagus plantlets, other than NJ362M, and male hybrids, for similar experiments described in previous chapters.

2. Field plot protection tests.
3. Studies to maximize the most effective combination of avirulent *F. oxysporum* (AVFO) and *F. solani* isolates to obtain maximum protection of asparagus plantlets against *F. oxysporum* or *F. moniliforme* alone or in combination.
4. Research into possible toxins produced by different *Fusarium* species, and their roles during infection process.
5. Research into the biology, ecology and physiology of *Fusarium solani*, and its probable similarity to mycorrhizal fungi.
6. *In vitro* research into the infectivity of *Fusarium* species on asparagus plantlets at different inoculum concentrations under various environmental and nutritional conditions.
7. Use of immunological techniques such as monoclonal antibody and immunofluorescent microscopy to detect specific fungal hyphae, and to differentiate hyphae of different fusarial species within asparagus plant or plantlet tissues.
8. Molecular level study to differentiate, compare and characterize different virulent and avirulent *Fusarium* species.

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